

Monitoring of Plasmid-Encoded, Trimethoprim-Resistant Dihydrofolate Reductase Genes: Detection of a New Resistant Enzyme

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Using gene-specific radiolabeled probe DNAs, we analyzed 42 clinical bacterial isolates with high-level trimethoprim (Tp) resistance for the presence of a type I or a type II plasmid-specified dihydrofolate reductase (DHFR) gene. Plasmid DNA from 17 strains harbored a type I DHFR, whereas 11 isolates contained plasmids that harbored a type II DHFR structural gene. The plasmid DNAs from five strains appeared to hybridize with both type I and type II DHFR probe DNAs. In addition, eight isolates had type I resistance determinants integrated into the chromosomes, presumably on transposon 7 (Tn7). Among the strains analyzed in this survey, none of the chromosomally located, Tp-insensitive reductases were of the type II class. Both the plasmid and chromosomal DNAs of one isolate showed no homology with either the type I or type II DHFR probe DNA. The plasmid harbored by this strain encoded a "new" Tp-resistant enzyme that differed significantly, both in molecular weight and with respect to trimethoprim and methotrexate inhibition kinetics, from the previously characterized plasmid-associated dihydrofolate reductases.

R plasmid-mediated trimethoprim (Tp) resistance has been recognized since 1971 (14). The mechanism of plasmid-associated Tp resistance is unusual in that the R plasmids harbor the structural genes for dihydrofolate reductases (DHFRs), which are highly resistant to trimethoprim; hence, the target site rather than the antimicrobial agent is modified (4, 25).

Plasmid-specified DHFRs can be arbitrarily divided into two broad categories based on different levels of sensitivity to Tp and other related antifolate compounds (23). Type I reductases have 50% inhibitory concentrations for trimethoprim several thousand-fold higher than that required to inhibit the *Escherichia coli* chromosomal DHFR. So-called type II DHFRs are, for all practical purposes, insensitive to Tp, having 50% inhibitory concentrations several hundred-fold higher than those for the type I reductases. Several of these plasmid-encoded enzymes have been reasonably well characterized. It is known, for example, that the type I DHFR specified by plasmid R483 (11) and the type II enzyme encoded by plasmid R67 are very different enzymes; they differ antigenically and in subunit structure (15). Furthermore, preliminary evidence suggests they are unrelated at the level of their nucleotide base sequence (D. Stone, personal communication).

In most parts of the world, the proportion of

pathogens sensitive to trimethoprim has remained high, despite increased usage of trimethoprim-sulfonamide combinations over the past 12 years (2). However, there is cause for concern that this resistance picture is changing. For example, Datta et al. (12) have reported that the incidence of Tp resistance in enterobacteria causing infections in a London hospital increased from 5.6% in 1970 to 16% in 1979. The resistance was transferable in 23% of the Tp-resistant strains examined in this particular survey. Recently, in this country, two clinical isolates, an *E. coli* and a *Klebsiella pneumoniae* strain harboring Tp resistance R plasmids, have been implicated in a fatal bacteremia in a leukemia patient undergoing Tp-sulfamethoxazole prophylaxis (35). In addition, Towner (31) has reported that the proportion of enterobacterial isolates with high-level, nontransferable trimethoprim resistance isolated from the Nottingham area of Great Britain rose from 4.7% in 1978 to 22.9% in 1979.

Clearly, these changing trends in susceptibility to trimethoprim need to be monitored at the molecular level, particularly since this specific plasmid-associated resistance has only recently emerged to a clinically significant extent. Using the series of techniques outlined in this report, we have been able to detect, locate, and discriminate between the type I and type II DHFR

genes in a variety of clinical bacterial isolates with high-level trimethoprim resistance. This study presents the results of such a survey.

MATERIALS AND METHODS

Bacterial strains and plasmids. The clinical isolates used in this study are described in Table 1. *E. coli* K-12, C-600 and *E. coli* B, HB101 were also used (6). The recombinant plasmid pFE364 was constructed by inserting a 2.5-kilobase (kb) *Bam*HI-*Eco*RI fragment of plasmid R67 into pBR322 (10). Plasmid pTP9-1 was constructed by inserting a 9.8-kb *Eco*RI-*Hind*III fragment of plasmid R483 into pBR322 (pTP9-1 was a gift from T. J. Kwok, Cold Spring Harbor Laboratory). Plasmid pFE506 is a 7.3-kb derivative of ColE1::Tn7 generated by digestion of the original plasmid with *Eco*RI (15).

Construction of DNA probes. In the experiments in which restricted and electrophoresed plasmid fragments were analyzed, the probe for the type I DHFR gene consisted of the 9.8-kb *Eco*RI-*Hind*III restriction fragment from plasmid pTP9-1, whereas the DNA probe used to detect the type II DHFR gene consisted of the 2.5-kb *Bam*HI-*Eco*RI restriction fragment derived from plasmid pFE364.

Plasmid pFE506 was used as the probe for the type I DHFR structural gene in the colony hybridization experiments. Plasmid pFE506 consists of a 4.3-kb fragment of Tn7 linked to a 3.0-kb fragment of ColE1 (10).

Plasmid DNA extraction and gel electrophoresis. Cultures were inoculated in 0.5 to 1.0 ml of Mueller-Hinton broth and grown overnight in Eppendorf tubes. The alkaline extraction method of Birnboim and Doly (9) was used to lyse the cells. This was followed by

restriction digestion with *Eco*RI (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and electrophoresis of the entire sample on a 0.7% agarose slab gel for 2 h at 120 V. After electrophoresis, gels were stained in ethidium bromide and photographed on a shortwave transilluminator screen (Ultraviolet Products, Inc., San Gabriel, Calif.). Gels were photographed with a 15-cm ruler to allow a comparison of the DNA bands in the original gel with the bands in the autoradiogram.

Isolation of DNA fragments and nick translation. Plasmid DNA was digested with the appropriate restriction enzyme combinations (Bethesda Research Laboratories), and the fragments were separated electrophoretically on 0.7 or 1.0% low-melting-point agarose (Bethesda Research Laboratories). Double digestion with the enzymes *Bam*HI and *Eco*RI was used for plasmid pFE364, and *Hind*III and *Eco*RI were used for plasmid pTP9-1. The agarose sections containing the DNA fragments of interest were excised and melted by heating to 70°C for 5 min. One volume of Tris buffer (0.5 M, pH 8.0) was added at 37°C. The melted agarose solution was extracted twice with 1 volume of Tris-saturated phenol. The phenol was removed from the aqueous solution by several extractions with ether. DNA was precipitated at -20°C by adding NaCl to a final concentration of 0.1 M and 2 volumes of 95% ethanol. The fragments were radiolabeled by using 0.2 pmol [α -³²P]dCTP (specific activity >600 Ci/mmol, New England Nuclear Corp., Boston, Mass.) and DNA polymerase I (Boehringer Mannheim Corp., Indianapolis, Ind.) under the conditions described by Maniatis et al. (20).

DNA transfer to membrane filters. A modified Southern method of DNA transfer to membrane filters (27) was employed in which DNA was transferred

TABLE 1. Bacterial strains

Strain designation	Organism	Phenotype ^a	Geographical origin	Source/reference
P42	<i>Enterobacter cloacae</i>	Tp	Finland	K. Dornbusch
P62	<i>Citrobacter</i> sp. 2623	Ap Cm Sm Su Tc Tp	Finland	30
P63	<i>Proteus mirabilis</i> 2933	Sm Su Tc Tp	Finland	30
P64	<i>E. coli</i> 2950	Ap Cm Sm Su Tc Tp	Finland	30
P65	<i>Citrobacter</i> sp. 3028	Ap Sm Su Tc Tp	Finland	30
P100	<i>E. coli</i> J6-2 (R388)	Sm Su Tp Tra	United Kingdom	23
P193	<i>K. pneumoniae</i>	Ap Cm Km Sm Su Tc Tp Tra	United States	J. Acar
P195	<i>E. coli</i> C600 (R721) ^b	Sm Tp Tra	United Kingdom	7
P223	<i>E. coli</i> 71	Tp Tra	France	J. Acar
P237	<i>E. coli</i> J5-3 (R751) ^c	Tp Tra	United States	17
P258	<i>E. coli</i> 72	Cm Sm Su Tc Tp Tra	France	J. Acar
P259	<i>E. coli</i> 73	Ap Cm Km Sm Su Tc Tp Tra	France	J. Acar
P570	<i>Shigella sonnei</i>	Ap Cm Sm Su Tp Tra	Brazil	28
P590	<i>S. typhimurium</i>	Sm Tp Tra	United Kingdom	34
P626	<i>K. pneumoniae</i>	Ap Sm Su Tc Tp Tra	Nigeria	H. Bialy
P629	<i>E. coli</i>	Tc Tp Tra	Finland	K. Dornbusch
P669	<i>E. coli</i> 435 ^d	Su Tp	New Zealand	1
P759	<i>E. coli</i>	Km Sm Su Tp Tra	Mexico	21

^a Nomenclature is consistent with that suggested by Novick et al. (22). Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Tp, trimethoprim; Tra, mediating conjugation.

^b Plasmid contains TN7.

^c Plasmid contains TN402 (24).

^d *E. coli* J5-3 transconjugant; donor was *S. typhimurium* phage type 179 (1).

bidirectionally in the presence of 1 M ammonium acetate (18, 26). Nitrocellulose membranes (Schleicher & Schuell Co., Keene, N.H.) processed with ammonium acetate required either thorough air drying at 37°C or a distilled water rinse to prevent excessive tearing. Gene Screen membranes (New England Nuclear) were also air dried but were less fragile. DNA was fixed to the membranes by drying for 2 h at 80°C.

DNA hybridization and autoradiography. Filters were pretreated by enclosing them in a heat-sealable bag with a solution of 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 1× Denhardt solution (0.2% bovine serum albumin, polyvinyl pyrrolidone, and Ficoll [molecular weight, 400,000]) (13) for 3 h at 65°C. The prehybridization solution was then replaced by hybridization solution containing 0.2 µg of radiolabeled fragment per ml for 18 h at 65°C. The filters were then washed sequentially in 3× SSC, 1× SSC, and 0.1× SSC containing 0.1% sodium dodecyl sulfate. When dry, the filters were covered with plastic wrap and placed in contact with X-Omat AR X-ray film (Eastman Kodak Co., Rochester, N.Y.). The film was exposed for 1 to 5 days, using intensifier screens (Eastman Kodak) at -80°C.

Colony hybridization. The colony hybridization technique described by Thayer (29), with slight modifications (10, 16), was used. Bacterial colonies were lysed on nitrocellulose filters, and the released DNA was immobilized on filters. The hybridization mixture consisted of 6× SSC-FPB (0.15 M NaCl and 0.015 M sodium citrate, plus 0.2% concentrations each of Ficoll [molecular weight, 400,000], polyvinyl pyrrolidone, and bovine serum albumin), containing 90 µg of sheared calf thymus DNA per ml and 10⁵ cpm of probe DNA. Kodak X-Omat AR film was exposed to the dried filters for 20 to 48 h, using an intensifying screen at 70°C.

Isolation and characterization of DHFR. Enzyme was prepared from cells grown in Mueller-Hinton broth as described by Pattishall et al. (23), with the following modification: Ultrogel Aca54 was used for gel filtration chromatography in place of Sephadex G-75. DHFR activity was measured spectrophotometrically in 0.1 M imidazole chloride buffer (pH 7) (5) in the presence and absence of 50 nM trimethoprim to distinguish resistant and sensitive enzymes. The 50% inhibitory concentrations for methotrexate and trimethoprim were determined as previously described (5).

RESULTS

Detection of specific DHFR structural genes. Forty-two plasmid-containing, Tp-resistant clinical bacterial isolates were lysed according to the procedure outlined in Materials and Methods. The resultant purified plasmid DNAs were restricted with *EcoRI*, and the fragments were electrophoresed in a 0.7% agarose gel. Figure 1 is a photograph of such a gel containing the restricted extrachromosomal DNA extracted from 12 Tp-resistant strains. Nitrocellulose paper containing DNA fragments blotted from this gel was probed with [³²P]DNA containing the type II DHFR structural gene (Fig. 2). Seven of the 12 DNA samples tested hybridized with

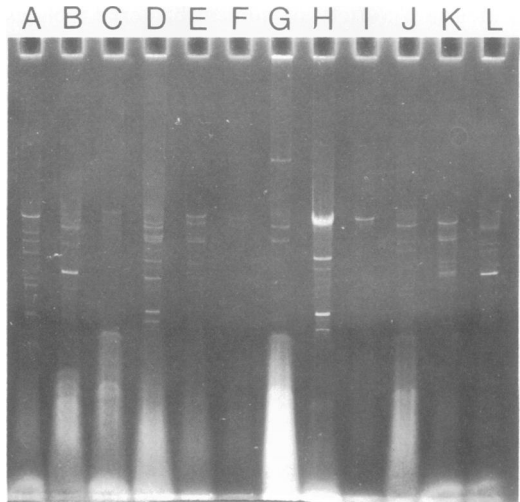


FIG. 1. Agarose gel in which plasmid DNAs extracted from Tp-resistant isolates were cleaved with *EcoRI* and electrophoresed. This gel corresponds to the autoradiograph shown in Fig. 2, in which the strain numbers are given.

radiolabeled type II DHFR probe. The remaining five DNAs showed no nucleotide sequence homology with this particular probe. The faint, common band (Fig. 2, arrow) is RNA. When samples were treated with RNase before electrophoresis, this band disappeared.

Figure 3 shows the results of a similar experiment in which a gel blot was probed with the ³²P-labeled DNA fragment containing the type I DHFR structural gene. Restricted plasmid DNAs that showed sequence homology with the type II DHFR probe (Fig. 2) did not react with radiolabeled type I probe DNA (e.g., P258, P259, P193, P570, P626, and P237). Conversely, strains whose restricted plasmid DNAs did not react with the type II DHFR DNA probe hybridized strongly with the radiolabeled type I DHFR DNA probe (e.g., P195, P629, P42, P590, and P759), indicating the high degree of specificity of the gene probes.

Table 2 shows the distribution of the type I and type II DHFR genes in the plasmid DNA extracted from 42 clinical isolates with high-level Tp resistance. Of particular interest was the fact that nine resistant strains harbored plasmid DNA that, upon restriction and electrophoresis, apparently did not contain complementary nucleotide sequences to either the type I or the type II DHFR probe. Five of these isolates originated in Turku, Finland. These multiply resistant Finnish strains all contained large plasmids; however, in all cases, Tp resistance was found to be nontransferable, although ampicillin and tetracycline resistance could be transferred from many of the isolates (J. Acar

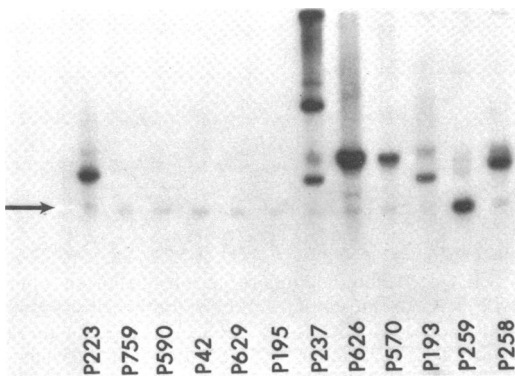


FIG. 2. Autoradiograph of an agarose gel containing *Eco*RI-generated restriction fragments from plasmids extracted from Tp-resistant clinical isolates. Gel was probed with a 32 P-labeled DNA fragment containing the structural gene for a type II DHFR. Strain phenotypes are given in Table 1.

and F. Goldstein, personal communication). In addition, Then and Hermann (30) reported that these strains synthesize an additional, Tp-insensitive reductase with an inhibitory profile similar to that of a plasmid-specified type I DHFR. Representative colonies of each of the nine "double-negative" strains were lysed on nitrocellulose filters, and the resultant whole cell DNA imprints were probed with 32 P-labeled plasmid pFE506. This derivative plasmid contains the structural gene for the type I DHFR harbored by Tn7 (8). The whole cell DNA from four of these Tp-resistant, "double-negative" isolates (P62-P65) hybridized with the type I DHFR probe (Fig. 4). A positive control strain (P195) gave an expected positive reaction. On the other hand, no reaction was observed between this probe and DNA extracted from a plasmid-free, Tp-sensitive strain (F^-) or an isolate harboring the structural gene for a type II DHFR (P100). Since plasmid pFE506 contains a fragment of *ColE1* (10), a colony hybridization experiment was carried out, using 32 P-labeled *ColE1* as a probe. None of the DNA imprints hybridized with radiolabeled *ColE1* (data not shown). These results suggest that Tn7, or at least a portion of Tn7 containing the structural gene for DHFR, has become integrated into the chromosome of these Tp-resistant strains. An alternative interpretation is that these isolates harbor a second, presumably larger, plasmid that is going undetected with the lysis conditions employed in this study. We consider this possibility to be extremely unlikely.

Restricted plasmid DNA from five resistant strains (Table 2) appeared to hybridize with both probe DNAs. In the case of one isolate, we have convincing evidence that the structural genes for both resistant DHFRs reside on the same plas-

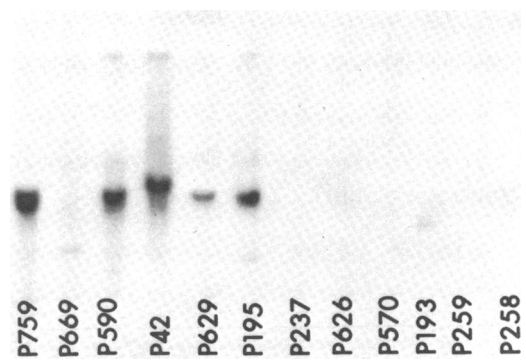


FIG. 3. Autoradiograph of an agarose gel containing *Eco*RI-generated restriction fragments from plasmids extracted from Tp-resistant clinical isolates. Gel was probed with a 32 P-labeled DNA fragment containing the structural gene for a type I DHFR. Strain phenotypes are given in Table 1.

mid. In the case of the other four strains, another explanation is more likely. The type I DHFR probe originally used in these experiments contained a 4-kb segment of Tn7 which included at least a portion of the gene specifying a streptomycin-inactivating enzyme. Therefore, the potential for cross-hybridization with non-DHFR genes is inherently greater with the type I probe compared with the type II DHFR probe. However, this ambiguity was a problem in only five (9.5%) of the isolates examined in this survey. One of us (M.F.) has constructed a type I probe consisting of a 1.0-kb *Taq*I fragment from plasmid R483. Our preliminary results show that plasmid DNA fragments from most of the "double-positive" strains do not react, or hybridize only weakly, with this smaller type I DHFR probe.

Detection of a "new" plasmid-specified DHFR. In the course of this survey, we encountered one Tp-resistant isolate (P669, Table 1) that was especially intriguing. This *E. coli* K-12 transconjugant harbored an 8-kb plasmid that conferred resistance to trimethoprim and sulfonamide (1). The donor was a strain of *Salmonella typhimurium* phage type 179, an organism implicated in epidemics in cattle and sporadic human infections in Great Britain (2, 34). The *Salmonella* donor strain contained two R plasmids: a 90-kb plasmid conferring ampicillin and tetracycline resistance and an 8-kb trimethoprim and sulfonamide resistance plasmid (1). This particular *E. coli* transconjugant (strain P669) harbored only the 8-kb plasmid. It was presumably mobilized from the donor by the 90-kb plasmid, which was subsequently eliminated from the recipient strain. Of further interest was the fact that the minimal inhibitory concentration (MIC) for trimethoprim in both the *S. typhimurium* and *E.*

TABLE 2. Distribution of the type I and type II DHFR genes in the plasmids extracted from 42 naturally occurring bacterial strains with high-level trimethoprim resistance

Radiolabeled probe DNA containing:		No. of strains reacting ^a
Type I DHFR gene	Type II DHFR gene	
+	+	5
+	-	17
-	+	11
-	-	9

^a Plasmid DNA was extracted, purified, and electrophoresed as described in Materials and Methods. Agarose gels containing plasmid fragments were probed with ³²P-labeled DNA fragments containing either the type I or the type II DHFR structural gene.

coli hosts that harbored this particular plasmid was 64 µg/ml. This is quite low compared with that of transferable Tp resistance previously observed, in which the MIC for trimethoprim was 1,000 to 2,000 µg/ml (23). Furthermore, restricted plasmid DNA isolated from the *E. coli* transconjugant strain P669 did not hybridize with the radiolabeled type I DHFR DNA probe (Fig. 3), nor did it react with the type II DHFR probe (data not shown). The possibility that either a type I or a type II Tp-resistant reductase gene had become incorporated into the chromosome of this strain was ruled out by the fact that colony hybridization experiments, using both radiolabeled DNA probes, were clearly negative (this was the only strain of nine "double-negative" isolates that did not react in the colony hybridization experiment).

The plasmid-specified reductase of this strain has been partially characterized (D. Baccanari, personal communication). It appears to be significantly different from both of the previously described plasmid-specified DHFR classes (23). For example, its molecular weight is 16,000 to 18,000, compared with a molecular weight of 34,000 to 36,000 for both the type I and type II reductases. The trimethoprim 50% inhibitory concentration for this "new" DHFR is approximately 1.5 µM, whereas the equivalent values for representative type I and type II DHFRs are 60 and 70,000 µM, respectively. In addition, this reductase is antigenically distinct from the DHFRs specified by plasmids R67 and R483 and from the chromosomal DHFR of *E. coli* RT500 (Sheila Smith, personal communication).

DISCUSSION

There is an accumulating body of evidence to suggest that the incidence of pathogenic bacteria with high-level, transferable trimethoprim resist-

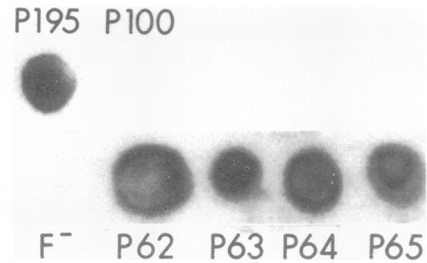


FIG. 4. Autoradiograph of a nitrocellulose filter with Tp-resistant colonies after colony hybridization with [³²P]DNA containing the structural gene for a type I DHFR. Strains P62, P63, P64, and P65 have high-level, nontransferable Tp resistance (see Table 1), contain extrachromosomal DNA, and synthesize a DHFR similar to the type I plasmid-encoded reductase (30). F⁻, *E. coli* C600; P195, *E. coli* C600 (R721)—plasmid R721 contains Tn7; P100, *E. coli* J6-2 (R388)—plasmid R388 harbors the structural gene for a type II DHFR (15).

ance is increasing, especially in the hospital environment (4, 32). The need to closely monitor developing trends in bacterial susceptibility to trimethoprim, as well as to other antimicrobial agents, is an obvious one.

Using the sequence of techniques outlined in this study, we screened 42 naturally occurring bacterial strains with high-level Tp resistance for the presence of either type I or type II plasmid-encoded reductase genes. The plasmid DNAs of 17 strains (40.5%) contained the structural gene for a type I reductase, whereas the chromosomal DNAs of eight isolates (19%) appeared to harbor the type I gene determinant. The predominance of the type I DHFR gene among these isolates (59.5% of the total analyzed, if the strains with type I DHFR determinants integrated into the chromosomes are included) can perhaps be explained by the fact that this determinant resides on a transposon, Tn7. The fact that almost one-fifth of the strains analyzed in this survey contained type I plasmid-encoded DHFR genes integrated into their chromosomes supports the recent report of a significant increase in the isolation frequency of clinical strains with high-level, nontransferable trimethoprim resistance (31). This is a potentially disquieting trend since this resistance is inherently more stable than plasmid-mediated resistance and not likely to be lost once the selective pressure is removed.

Lichtenstein and Brenner (19) have recently shown that Tn7 preferentially inserts into the *E. coli* chromosome at high efficiency, into a unique site, and with a single orientation. It should be noted, however, that we have not analyzed the site of Tn7 insertion into the chromosomes of any of these Tp-resistant clinical strains, nor do we know whether Tn7 exists as a

functional transposable element in any of these replicons, plasmid or chromosome.

Plasmid DNAs of 11 strains (26%) harbored the structural gene for a type II reductase. Although it has been shown that the Tp resistance transposon 402 (Tn402) contains a gene encoding a type II DHFR (15), it is not known whether any of the type II DHFR determinants detected among the strains in this survey are transposable. What can be said with certainty is that none of the chromosomally located, Tp-insensitive reductases were of the type II class.

Towner and Pinn (33) have recently reported a transferable plasmid, pUN212, harbored by a clinical isolate of *E. coli*, that conferred a moderate level of resistance to trimethoprim (MIC = 256 µg/ml) compared with the level of resistance usually associated with R plasmids (MIC greater than 1 mg/ml). These authors speculated that this R plasmid specified an "intermediate" type of DHFR more sensitive to trimethoprim than the "normal" plasmid-encoded reductases. The enzyme specified by plasmid pUN212 was not characterized. We found and partially characterized such an "intermediate" plasmid-specified DHFR originating in a multiply drug-resistant *S. typhimurium* strain isolated by Anderson in New Zealand (1). This unusual reductase differs significantly from its plasmid-associated predecessor DHFRs in molecular weight and inhibition profile (Tp resistant and yet methotrexate sensitive), as well as the relatively low level of trimethoprim resistance it confers upon its host bacterium (64 µg/ml). This finding serves to deepen the mystery as to the origin of these resistance determinants (e.g., the amino acid sequence of the type II plasmid-encoded DHFR bears no sequence homology with any other characterized reductase: bacterial, phage, or mammalian) and suggests that these plasmid-specified enzymes comprise a rather heterogeneous group.

ACKNOWLEDGMENTS

We thank the following people for supplying us with bacterial strains: J. Acar, D. Anderson, H. Bialy, K. Dornbusch, B. Murray, B. Rowe, D. Taylor, and G. White. In addition, we are grateful to David Baccanari (Microbiology Department, Wellcome Research Laboratories) for doing enzyme analyses on selected isolates.

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