ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1992, p. 1720–1726
0066-4804/92/081720-07$02.00/0

Vol. 36, No. 8

Identification by DNA Sequence Analysis of a New Plasmid-Encoded Trimethoprim Resistance Gene in Fecal *Escherichia coli* Isolates from Children in Day-Care Centers

KAVINDRA V. SINGH,[1] RANDALL R. REVES,[1,2] LARRY K. PICKERING,[3] AND BARBARA E. MURRAY[1,2,4]*

Center for Infectious Diseases[1] and Departments of Medicine,[2] Microbiology,[4] and Pediatrics,[3] University of Texas Medical School at Houston, Houston, Texas 77030

Received 3 January 1992/Accepted 26 May 1992

In our ongoing studies of trimethoprim resistance (Tmp^r) in day-care centers (DCC), we have shown a high rate of fecal colonization with Tmp^r *Escherichia coli* and, using total plasmid content analysis, have shown that this is due to a diversity of strains. In the present study, we analyzed 367 highly Tmp^r (MIC, ≥2,000 μg/ml) isolates of *E. coli* from 72 children over a 5-month period and found at least 83 distinct plasmid patterns, indicating that at least 83 strains were involved. Several strains were particularly common in a given DCC, including one found in 61% of children with Tmp^r *E. coli*; these common strains usually persisted within a DCC for several months. Colony lysates were hybridized with gene probes for dihydrofolate reductases (DHFR) types I, II, III, V, and VII; 21% hybridized under stringent conditions, and all of these were with type I (17%) or type V (4%) probes. Tmp^r was cloned from a probe-negative Tmp^r transconjugant, and an intragenic probe was prepared from this clone. Approximately 21% of the Tmp^r *E. coli* strains (76 isolates) in the DCC were found to have this new gene, 74 of which were in one DCC. The DNA sequence of this gene was determined, and the predicted amino acid sequence was shown to have between 32% and 39% identity with the amino acid sequences for types I, III, V, VI, and VII and the partial sequence of type IV and ~26% identity with types IX and X DHFR. This confirms the uniqueness of this gene, which has tentatively been named *dhfrxii*, and its translation product, DHFR type XII.

Trimethoprim (TMP) is a widely used antimicrobial agent. Following the introduction of TMP and TMP-sulfamethoxazole (SXT), TMP-resistant bacterial pathogens emerged as a clinical problem. The emergence of high levels of TMP and SXT resistance among *Escherichia coli* isolates from stool and urine is well reported in the literature, particularly in developing countries and in day-care centers (DCC) (15, 17, 18, 21, 22). In the United States, we have found that children in DCC caring for large numbers of diapered children have much higher rates of resistance (48% with Tmp^r *E. coli*) than children in smaller DCC (17%), children not in DCC (6%), or healthy adults (8%) (21). The present study was designed to evaluate the genetic diversity of Tmp^r among fecal *E. coli* isolates from children attending DCC in Houston, Tex.

MATERIALS AND METHODS

The DCC were enrolled in prospective studies of diarrhea and were selected for the current 5-month study from a group of 12 DCC (no. 1 through 12) that had been previously studied. Written informed consent from each DCC director and permission from the parents of the enrolled children were obtained. This study was approved by the Committee for the Protection of Human Subjects of the University of Texas Medical School at Houston (21).

Sample collection, processing, and media. Fecal samples from children enrolled in four DCC were collected in plastic containers and transported to the laboratory in styrofoam coolers for same-day plating on selective agar media by two methods. (i) The dilution method consisted of the following. About 1.5 mg of each stool specimen was suspended in 1.5 ml of 0.9% NaCl. After vortexing, a loopful (~1.5 μl) was streaked for single-colony isolation onto PW agar (32) containing 50 μg of TMP per ml, MacConkey agar containing 50 μg of TMP per ml, and PW agar with no drug. (ii) The direct streak method consisted of the following. Approximately 1.5 mg of each stool specimen was also streaked directly onto the media mentioned above. All inoculated plates were incubated at 37°C for 18 to 24 h. When available, five *E. coli*-like lactose-fermenting colonies and two non-lactose-fermenting colonies were picked from PW agar containing TMP plates (50 μg/ml) and saved in peptone stabs for further studies; non-lactose fermenters were included because our previous work had identified a number of Tmp^r *E. coli* isolates as being non-lactose fermenters.

Plasmid profiles, organism identification, susceptibility testing, and colony lysates. Over 900 Tmp^r colonies were collected for plasmid pattern analysis by the lysate method of Kado and Liu (13). Those colonies showing plasmid patterns distinct from each other were identified by the API-20E system (Analytical Products, Plainview, N.Y.). Plasmid patterns that were the same except for one or two (when more than five total plasmids were present) plasmids were considered to be the same pattern and not distinct patterns; this interpretation might underestimate the number of different patterns and thus the number of distinct strains. Susceptibility to TMP, sulfisoxazole (SU), SXT, ampicillin (AMP), cephalothin (CEP), chloramphenicol (CHL), tetracycline (TE), streptomycin (STR), and gentamicin (GM) was determined by the disk diffusion method with Mueller-Hinton

* Corresponding author.

TABLE 1. Plasmid and resistance patterns of Tmp^r *E. coli* from DCC

DCC no.	Total no. of diapered children	Total no. of plasmid patterns/total no. of colonies	Most common plasmid pattern[a] (% of children colonized)	Resistance patterns of common strains	Persistence of common pattern in DCC (mo)
4 (large)	26	22/148	11-00 (61)	AMP, CEP, STR, SXT, SU, TE, TMP	5
			01-00 (35)	AMP, CEP, STR, SXT, SU, TMP	5
5 (large)	28	35/158	08-00 (39)	CEP, STR, SXT, SU, TMP	5
			11-99 (11)	AMP, CHL, STR, SXT, SU, TE, TMP	2.5
1 (small)	8	16/43	No common plasmid pattern	Many[b]	—[c]
2 (small)	10	10/18	No common plasmid pattern	Many[b]	—[c]

[a] A numerical code system was used to name the different plasmid patterns.
[b] All strains were resistant to a minimum of three agents (AMP, SU, and TMP), and most were also resistant to other agents.
[c] No common pattern was found.

agar. National Committee for Clinical Laboratory Standards (19) criteria were used to classify strains as susceptible or resistant. High-level TMP resistance, defined as a MIC of ≥2,000 μg/ml), was determined by agar dilution.

A total of 367 highly Tmp^r colonies were inoculated onto MacConkey agar plates with a Steers replicator and were incubated overnight at 37°C. Colony lysates were made with Whatman 541 paper according to the method described by Maas (14). During the first part of the study, colony lysates were prepared only from Tmp^r isolates which had been shown to have distinct plasmid patterns; during the latter part of the study, colony lysates were prepared prior to plasmid pattern detection and represented five lactose-fermenting and two non-lactose-fermenting Tmp^r isolates (when present) per specimen. Results are reported only for isolates subsequently shown to be *E. coli*.

DNA labelling and hybridization. The type I dihydrofolate reductase (DHFR) probe was the 500-bp *Hpa*I fragment of plasmid pFE872 (8) provided by Mary Fling. The type II probe (provided by Susan Lester) was a *Pfl*MI-*Sfa*NI fragment isolated from the 850-bp *Eco*RI fragment of p700 (originally from R67) (9); this fragment had been blunt ended and cloned into the *Sma*I site of pUC19 and was regenerated with *Eco*RI-*Bam*HI double digestion. The type III DHFR probe was an 855-bp *Eco*RI-*Hin*dIII fragment of pFE1242 (7) (M. Fling). The type V probe was a 489-bp *Kpn*I-*Bam*HI fragment of plasmid pLK022 (26), and the type VII probe was a 314-bp *Eco*RV fragment of pLK0221A (both provided by Ola Skold). The probe for the new type XII gene was a 100-bp *Alu*I fragment (see Fig. 3). All DNA probes were purified from low-gelling-temperature agarose with GENECLEAN (Bio 101, Inc., La Jolla, Calif.) and labelled by random priming with [^{32}P]dCTP (5, 6). Hybridization was performed under stringent conditions (50% formamide and 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 42°C for 18 h), and the colony lysate filters were washed four times by using 2× SSC at room temperature with slow shaking (31). The agarose gels were alkali blotted to transfer restriction digestion DNA fragments onto Hybond-N membranes according to the instructions of the manufacturer (Amersham, Arlington Heights, Ill.).

Mating and transconjugant selection. Mating experiments (16) were performed with *E. coli* C600 (nalidixic acid and rifampin resistant) as the recipient strain and Mueller-Hinton agar supplemented with TMP (20 μg/ml) and nalidixic acid (50 μg/ml) for selection.

Cloning and subcloning of plasmid DNA. Wild-type plasmid DNA was collected by the method of Currier and Nester (4), digested with restriction enzymes, and ligated into vectors already cut with the same enzyme. In some cases, specific bands were excised from the low-gelling-temperature agarose and were used for ligation with the vector. The vectors pACYC184, pUC18, and pUC19 were used as the cloning vehicles. Ligations were followed by transformation into competent cells. Selections were made on Mueller-Hinton agar plus TMP (50 μg/ml) for Tmp^r clones and Luria-Bertani agar plus AMP (50 μg/ml) with isopropyl-β-D-thiogalactopyranoside (20 μl; 100 mM) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (50 μl; 2%) for TMP-susceptible clones. The restriction endonucleases, T4 DNA ligase, and HB101-competent *E. coli* cells were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), SURE cells were purchased from Stratagene (La Jolla, Calif.), and they were used according to the manufacturer's instructions.

DNA sequencing. DNA sequencing was performed by the chain termination method as described by Toneguzzo et al. (29) with the Sequenase sequencing kit and the M13 Sequencing Primer (−40) from U.S. Biochemicals Corp., Cleveland, Ohio, and the M13 Reverse Sequence Primer from Pharmacia LKB Biotechnology, Piscataway, N.J. The insert contained in pBEM155 was sequenced in both directions. The upstream region (to the right of the *Nde*I site in Fig. 3) was sequenced in only one direction. Sequence analysis was performed with MacVector (IBI, New Haven, Conn.) and the University of Wisconsin Genetics Computer Group software package on a VAX/VMS.

Nucleotide sequence accession number. The accession number M84522 has been assigned by GenBank.

RESULTS

Plasmid profile studies. Over 900 Tmp^r isolates collected over a 5-month period from children in four DCC were analyzed by gel electrophoresis to determine their plasmid profiles. Among the 26 children in DCC no. 4, there were 22 distinct plasmid pattern groups, which we interpret as indicating 22 distinct strains (Table 1); these were all identified as *E. coli*. The representative plasmid patterns are shown in Fig. 1 and 2. The plasmid patterns designated 01-00 and 11-00 seen in Fig. 1, lanes a and b, respectively, were found to be the two most common patterns in DCC no. 4 and were found repetitively in this DCC throughout the 5 months of the study. Both plasmid patterns shared common antibiotic resistance to TMP, SU, SXT, AMP, CEP, and STR, and isolates with pattern 11-00 also had resistance to TE. In the

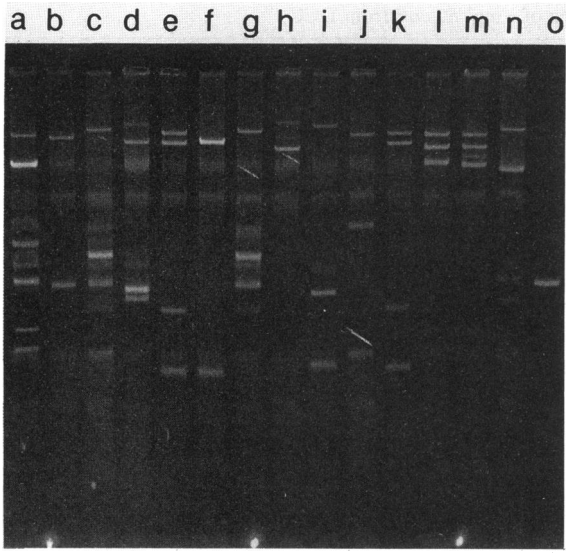

FIG. 1. Agarose gel electrophoresis showing distinct plasmid patterns of Tmp^r *E. coli* from DCC no. 4. Lanes a and b show the most common patterns, 01-00 and 11-00, respectively.

other large DCC (no. 5), there were 35 distinct plasmid patterns, all representing *E. coli* isolates. Representative plasmid patterns can be seen in Fig. 2. Two patterns, 08-00 and 11-99, shown in Fig. 2, lanes f and o, were found to be the most common patterns in DCC no. 5, and the colonies bearing these patterns were non-lactose-fermenting *E. coli* isolates. Isolates with pattern 11-99 showed resistance to CHL, TE, and AMP, as well as to TMP, SU, SXT, and STR; the latter resistance pattern was also observed in pattern 08-00 (Table 1). In the small DCC (no. 1 and 2), 16 and 10 distinct plasmid patterns were observed, respectively, with no predominant pattern. All isolates shared antibiotic resistance to TMP, SU, SXT, and AMP.

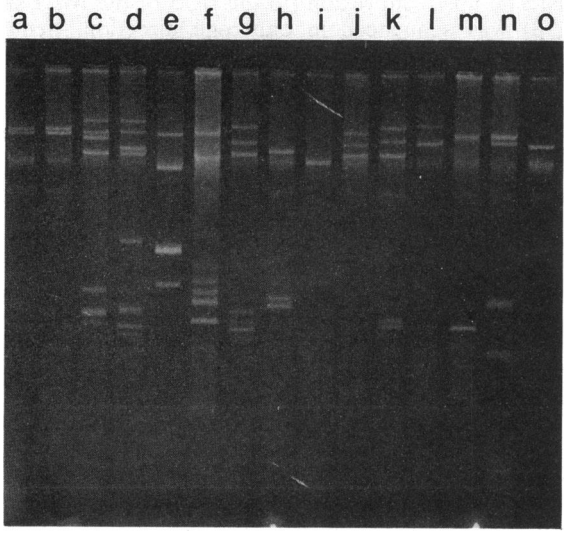

FIG. 2. Agarose gel electrophoresis showing distinct plasmid patterns of Tmp^r *E. coli* from DCC no. 5. Lane f shows one of the common strains, with plasmid pattern 08-00.

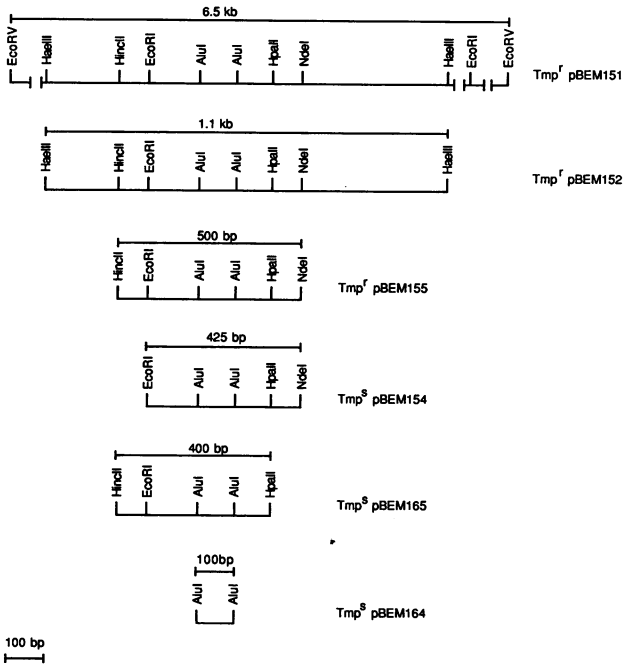

FIG. 3. Restriction endonuclease maps of TMP-resistant and TMP-susceptible clones. The 100-bp *Alu*I intragenic fragment of pBEM164 was used as a probe; two *Alu*I sites found by DNA sequence analysis between the *Hin*cII and *Eco*RI sites are not shown. Details are described in the text.

DNA labelling and hybridization. Among the 367 Tmp^r *E. coli* isolates from which colony lysates were prepared and hybridized with probes for Tmp^r genes, there were at least 83 distinct plasmid patterns and 13 isolates with no plasmids. DNA present in colony lysates of 61 *E. coli* isolates (16.6%) showed homology with the type I DHFR probe; these probe-positive isolates were found in 24 children (33%) and in each DCC. DNA in colony lysates from 14 *E. coli* isolates (3.8%) hybridized to the type V probe, including 2 isolates from one child (variants of the same plasmid pattern) which hybridized to the type I probe as well. A total of 14 type V-positive *E. coli* isolates were present in a total of seven children, all in DCC no. 5. None of the *E. coli* colony lysates showed hybridization with DHFR type II, III, and VII DNA probes.

Mating and transconjugant selection. A total of 34 of 80 bacteria (representing all four DCC) transferred Tmp^r to *E. coli* C600. One of the two predominant strains (08-00) in DCC no. 5 (Fig. 2, lane f) was able to transfer resistance (data not shown), while the other three predominant strains (11-99, 11-00, and 01-00) were not able to transfer resistance.

Cloning and subcloning of plasmid DNA. One Tmp^r *E. coli* isolate which had a transferable plasmid encoding high-level Tmp^r and which did not hybridize with any of the DHFR DNA probes tested, even under conditions of low stringency, was selected for further study. Initial cloning with *Bam*HI yielded Tmp^r recombinants with a 7.6-kb insert. A 6.5-kb *Eco*RV fragment was subcloned from this clone into the *Sma*I site of pUC18 to generate pBEM151 (Fig. 3). A restriction map with *Hae*III, *Hin*cII, *Eco*RI, *Hpa*II, and *Nde*I was generated, and these restriction sites were used to create a series of subclones in order to localize the Tmp^r gene. The two *Alu*I sites shown were mapped with

TABLE 2. Relatedness of the amino acid sequences of chromosomal and of TMP-resistant DHFRs

DHFR	% Similarity (identity)[a]								
	New	Type I	Type III	Type V	Type VI	Type VII	*E. coli*	*E. faecium*	*S. aureus*
New[b]	100	56 (32)	60 (35)	59 (39)	57 (35)	59 (39)	57 (36)	55 (30)	56 (34)
Type I[b]		100	57 (33)	84 (75)	75 (62)	81 (72)	58 (31)	52 (33)	58 (34)
Type III[b]			100	60 (34)	55 (33)	58 (33)	69 (51)	60 (34)	61 (41)
Type V[b]				100	73 (61)	78 (66)	62 (36)	55 (35)	56 (31)
Type VI[b]					100	75 (64)	58 (31)	56 (31)	55 (31)
Type VII[b]						100	59 (32)	50 (32)	58 (31)
E. coli K-12[c]							100	61 (33)	60 (37)
E. faecium[c]								100	64 (34)
S. aureus S1[b]									100

[a] Relatedness determined by the GAP alignment program of the University of Wisconsin Genetics Computer Group software package for VAX/VMS computers.
[b] Encodes TMP resistance.
[c] Chromosomal DHFR.

pBEM154; two *Alu*I sites outside this clone (between the *Hin*cII and *Eco*RI sites) were determined by sequence analysis but are not shown on this figure. As can be seen in Fig. 3, pBEM155, containing an ~500-bp *Nde*I-*Hin*cII fragment, was the smallest clone which expressed high-level TMP resistance. The lack of resistance in clones pBEM154 and pBEM165 suggests that both ends of pBEM155 are essential for the expression of TMP resistance. A 100-bp *Alu*I fragment from pBEM155 was then cloned into the *Hin*cII site of pUC18, and the resultant plasmid was designated pBEM164 (Fig. 3). A probe from this fragment did not hybridize to colony lysates of strains containing genes for known type I, II, III, V, or VII DHFR. This probe was tested against the 367 Tmp^r clinical isolates from the four DCC and found to hybridize with 76 *E. coli* isolates (21%) from 22 children (31%); 74 of these isolates were from DCC no. 4, representing 19 different plasmid patterns or variants and 2 strains with no plasmids. Of the two other isolates, one was from DCC no. 5 and one was from DCC no. 1. The large number of organisms positive with this probe in DCC no. 4 indicates spread of this new TMP resistance gene to various strains with different plasmid patterns.

Sequence analysis. The DNA sequence of a 714-nucleotide (nt) region which contained an open reading frame of 495 nt was determined; the putative translation product of 165 amino acids is shown in Fig. 4, which is reversed relative to the inserts in Fig. 3. Analysis of the sequence upstream of the putative ATG start codon did not reveal a typical *E. coli* promoter sequence or ribosomal binding site. The gene also generated resistance even when cloned with the *Nde*I recognition site, which includes the putative start codon, suggesting that it can utilize sequences in the vector to promote transcription and initiate translation. There were several other open reading frames in this region, but all were ≤195 nt (65 amino acids). The conclusion that the translation product of the 495-nt open reading frame is a DHFR is strengthened by the results of a search for related sequences in the Protein Information Resource data base of the Genetics Computer Group package with the program FASTA. The first 16 matches were all bacterial DHFRs, including *E. coli* types I and V DHFRs, the type III DHFR from *Salmonella typhimurium*, the *E. coli* K-12 chromosomal DHFR, and a plasmid-encoded DHFR causing TMP resistance in *Staphylococcus aureus*, as well as DHFRs from other bacterial species. The next 19 matches were to the DHFRs of eukaryotes. The sequences of DHFR types VI, IX, and X were not yet in this data base or two other data bases (GenBank, release 69; EMBL, release 28; Protein Information Resource-Protein, release 29).

Figure 5 shows the amino acid sequence deduced from the DNA sequence aligned by the GAP program of the Genetics Computer Group package to the amino acid sequences of several well-characterized DHFRs (7, 8, 23, 24). The similarity (based on conserved amino acid changes) and identity, respectively, of the new protein's sequence to the sequences of these other DHFRs were 56% and 32% for type I, 60% and 35% for type III from *S. typhimurium*, 59% and 39% for type V, and 57% and 36% for the *E. coli* K-12 chromosomal DHFR. The amino acid residues previously shown to be identical in the DHFRs from diverse species (7, 30) were also present in this sequence and are marked with an asterisk. The type II DHFRs, which do not share these amino acids, had no significant homology to the new DHFR.

Table 2 shows the relationships of the DHFRs aligned in Fig. 5 to the DHFR of *Streptococcus* (*Enterococcus*) *faecium*, a plasmid-encoded Tmp^r DHFR from *S. aureus* (both obtained through GenBank), the recently published sequence of the type VI DHFR from *Proteus mirabilis* (33), and the unpublished sequence for DHFR type VII (obtained from Ola Skold) (25). The new protein was also found to have 46% similarity and 26% identity with the recently reported type X DHFR (20) and 46% similarity and 27% identity with the type IX DHFR (12). Compared with the N-terminal amino acid sequence of the type IV protein (28), the new sequence had 61% similarity and 33% identity in this region. The N-terminal amino acid region reported from a plasmid-encoded DHFR from *Shigella sonnei* (27) had 57% similarity and 35% identity with this part of our new sequence; the *S. sonnei* protein has been referred to as type IIIb (3) but has only 50% similarity and 39% identity with the original type III DHFR from *S. typhimurium* (7, 27) and, thus, should probably not be considered a variant of type III.

DISCUSSION

Considerable attention has been given to antibiotic resistance found among pathogenic strains of bacteria causing disease in humans, animals, and plants. However, less attention has been given to the prevalence of these resistance genes in the fecal flora of humans at large, which may be a harbinger of resistance in strains causing infections. In this and our earlier studies, TMP resistance has been used to investigate the resistance of fecal flora in the pediatric age group. As before (22), we found a diversity of plasmids

```
                    20                  40                  60                  80
TCACGCAACTGGTCCAGAAC CTTGACCGAACGCAGCGGTG GTAACGGCGCAGTGGCGGTT TTCATGGCTTGTTATGACTG
AGTGCGTTGACCAGGTCTTG GAACTGGCTTGCGTCGCCAC CATTGCCGCGTCACCGCCAA AAGTACCGAACAATACTGAC

                   100                 120                 140                 160
TTTTTTTGTACAGTCTATGC CTCGGGCATCCAAGCAGCAA GCGCGTTACGCCGTGGGTCG ATGTTTGATGTTATGGAGCA
AAAAAAACATGTCAGATACG GAGCCCGTAGGTTCGTCGTT CGCGCAATGCGGCACCCAGC TACAAACTACAATACCTCGT

                   180                 200                 220
GCAACGATGTTACGCAGCAG GGCAGTCGCTAAACAAGTAG CAT ATG AAC TCG GAA TCA GTA CGC ATT TAT CTC
CGTTGCTACAATGCGTCGTC CCGTCAGCGATTTGTTCATC GTA TAC TTG AGC CTT AGT CAT GCG TAA ATA GAG
                                           Met Asn Ser Glu Ser Val Arg Ile Tyr Leu

      240                 260                 280
GTT GCT GCG ATG GGA GCC AAT CGG GTT ATT GGC AAT GGT CCT AAT ATC CCC TGG AAA ATT CCG
CAA CGA CGC TAC CCT CGG TTA GCC CAA TAA CCG TTA CCA GGA TTA TAG GGG ACC TTT TAA GGC
Val Ala Ala Met Gly Ala Asn Arg Val Ile Gly Asn Gly Pro Asn Ile Pro Trp Lys Ile Pro

  300                 320                 340                 360
GGT GAG CAG AAG ATT TTT CGC AGA CTC ACT GAG GGA AAA GTC GTT GTC ATG GGG CGA AAG ACC
CCA CTC GTC TTC TAA AAA GCG TCT GAG TGA CTC CCT TTT CAG CAA CAG TAC CCC GCT TTC TGG
Gly Glu Gln Lys Ile Phe Arg Arg Leu Thr Glu Gly Lys Val Val Val Met Gly Arg Lys Thr

                    380                 400                 420
TTT GAG TCT ATC GGC AAG CCT CTA CCG AAC CGT CAC ACA TTG GTA ATC TCA CGC CAA GCT AAC
AAA CTC AGA TAG CCG TTC GGA GAT GGC TTG GCA GTG TGT AAC CAT TAG AGT GCG GTT CGA TTG
Phe Glu Ser Ile Gly Lys Pro Leu Pro Asn Arg His Thr Leu Val Ile Ser Arg Gln Ala Asn

                  440                 460                 480
TAC CGC GCC ACT GGC TGC GTA GTT GTT TCA ACG CTG TCG CAC GCT ATC GCT TTG GCA TCC GAA
ATG GCG CGG TGA CCG ACG CAT CAA CAA AGT TGC GAC AGC GTG CGA TAG CGA AAC CGT AGG CTT
Tyr Arg Ala Thr Gly Cys Val Val Val Ser Thr Leu Ser His Ala Ile Ala Leu Ala Ser Glu

                500                 520                 540
CTC GGC AAT GAA CTC TAC GTC GCG GGC GGA GCT GAG ATA TAC ACT CTG GCA CTA CCT CAC GCC
GAG CCG TTA CTT GAG ATG CAG CGC CCG CCT CGA CTC TAT ATG TGA GAC CGT GAT GGA GTG CGG
Leu Gly Asn Glu Leu Tyr Val Ala Gly Gly Ala Glu Ile Tyr Thr Leu Ala Leu Pro His Ala

            560                 580                 600
CAC GGC GTG TTT CTA TCT GAG GTA CAT CAA ACC TTC GAG GGT GAC GCC TTC TTC CCA ATG CTC
GTG CCG CAC AAA GAT AGA CTC CAT GTA GTT TGG AAG CTC CCA CTG CGG AAG AAG GGT TAC GAG
His Gly Val Phe Leu Ser Glu Val His Gln Thr Phe Glu Gly Asp Ala Phe Phe Pro Met Leu

          620                 640                 660
AAC GAA ACA GAA TTC GAG CTT GTC TCA ACC GAA ACC ATT CAA GCT GTA ATT CCG TAC ACC CAC
TTG CTT TGT CTT AAG CTC GAA CAG AGT TGG CTT TGG TAA GTT CGA CAT TAA GGC ATG TGG GTG
Asn Glu Thr Glu Phe Glu Leu Val Ser Thr Glu Thr Ile Gln Ala Val Ile Pro Tyr Thr His

    680                 700
TCC GTT TAT GCG CGT CGA AAC GGC TAA CCA TTC CGT CGA C
AGG CAA ATA CGC GCA GCT TTG CCG ATT GGT AAG GCA GCT G
Ser Val Tyr Ala Arg Arg Asn Gly  *
```

FIG. 4. DNA sequence and translation of a 714-nt region and a translation product of 165 amino acids.

```
              1           *     * *     **          *           *50
DHFR-new      MNSESVRIYL VAAMGANRVI GNGPNIPWKI PGEQKIFRRL TEGKVVVMGR
     Type I   .....MKLSL MVAISKNGVI GNGPDIPWSA KGEQLLFKAI TYNQWLLVGR
     Type V   .....MKVSL MAAKAKNGVI GCGPHIPWSA KGEQLLFKAL TYNQWLLVGR
     Type III .....MLISL IAALAHNNLI GKDNLIPWHL PADLRHFKAV TLGKPVVMGR
E. coli chrom .....MISL IAALAVDRVI GMENAMPWNL PADLAWFKRN TLNKPVIMGR

            51*       *   *                                   100
DHFR-new      KTFESIGKPL PNRHTLVISR QANYRATGCV VV.STLSHAI ALASELGNEL
     Type I   KTFLSMG.AL PNRKYAVVTR SSFTSDNENV LIFPSIKDAL TNLKKITDHV
     Type V   KTFLSMG.AL PNRKYAVVTR SAWTADNDNV IVFPSIEEAM YGLAELTDHV
     Type III RTFESIGRPL PGRRNVVVSR NP.QWQAEGV EVAPSLDAAL ALLTDCEEAM
 E. coli chrom HTWESIGRPL PGRKNIILSS QP..GTDDRV TWVKSVDEAI AACGDVPEIM

           101 **                                            150
DHFR-new      YVAGGAEIYT LALPHAHGVF LSEVHQTFEG DAFFPMLNET EFELVSTET.
     Type I   IVSGGGEIYK SLIDQVDTLH ISTIDIEPEG DVYFPEI.PS NFRPVFTQ..
     Type V   IVSGGGEIYR ETLPMASTLH ISTIDIEPEG DVFFPNI.PN TFEVVFEQ..
     Type III II.GGGQLYA EALPRADRLY LTYIDAQLNG DTHFPDYLSL GWQELERSTH
E. coli chrom .VIGGGRVYE QFLPKAQKLY LTHIDAEVEG DTHFPDYEPD DWESVFSEFH

              151                 171
DHFR-new      ...IQAVIPY THSVYARRNG
     Type I   ..DFASNINY SYQIWQKG
     Type V   ..HFSSNINY CYQIWQKG
     Type III PADDKNSYAC EFVTLSRQR
E. coli chrom DADAQNSHSY CFEILERR
```

FIG. 5. Alignment of the sequence of the new DHFR putative type XII with those of types I, III, and V DHFRs and the chromosomal (chrom) DHFR from *E. coli* K-12. Twenty-six amino acids were identical in all five of these proteins (shaded residues); the 12 amino acid residues marked with an asterisk had been previously shown to be identical in the DHFRs from diverse species (7, 30).

among Tmp^r *E. coli*, including 57 distinct plasmid patterns among Tmp^r *E. coli* in the two large DCC (no. 4 and 5) and 26 distinct plasmid patterns in the two small DCC (no. 1 and 2). Since plasmid patterns that appeared similar (±1 to 2 plasmids [see above]) were classified as being the same plasmid pattern and since we used distinct plasmid patterns to classify organisms as different strains, the presence of 83 clearly distinct plasmid patterns indicates that a minimum of 83 different Tmp^r strains were present. All isolates were multiresistant, with resistance most often to SU, SXT, beta-lactams, STR, and TE.

The findings in the present study are significant not only because they again illustrate the diversity of strains colonizing diapered children in DCC settings with Tmp^r *E. coli* but also because we have documented the existence of a new TMP resistance gene encoding high-level TMP resistance. A number of DHFRs which confer high-level resistance have been described, including types I, V, VI, and VII (Table 2) and the unrelated type II (1, 2, 10, 11, 24, 25, 33). The newly described type X DHFR confers a MIC of 500 μg/ml (20). The type III DHFR from *S. typhimurium* and the type IX DHFR (found in swine) confer low-level resistance (MICs, 64 to 256 μg/ml), unlike our gene (MIC, ≥2,000 μg/ml), and the type IV DHFR generates even lower MICs (MICs, 5 to 40 μg/ml in Mueller-Hinton agar) (34). In the present study, all of the test bacteria were highly resistant to TMP (MIC,

≥2,000 μg/ml) and were found to be negative with type II and VII DNA probes; ~17% and ~4% isolates showed homology with type I and V DHFR probes, respectively. An additional 21% of isolates showed homology to the newly constructed intragenic probe in this study. The gene for type III DHFR was not found, but neither the so-called IIIb nor IIIc (which also encode low-level resistance) was tested; published probes for these genes are quite large (1,600 to 1,800 nt), with a probable gene size of ~500 nt. It is not known whether the remaining probe-negative colonies (58%) have another new gene or whether they have type VI or perhaps a variant of type III, IV, IX, or X which is able to confer high-level resistance.

Among the different gram-negative DHFRs whose entire sequences are known, types I, V, VI, and VII are most closely related (61% to 75% identity) while types III, IX, and X, the *E. coli* K-12 chromosomal DHFR, and our putative new DHFR are more distantly related to these four genes and each other, having from 26% to 39% identity; the type II DHFR is unrelated to these other DHFRs. In order to compare these numbers with the relatedness of other antimicrobial resistance genes, the GAP program was also applied to several β-lactamases; the percent similarity and percent identity of TEM-1 to other class A β-lactamase are, respectively, 99% and 99% for TEM-2, 80% and 68% for SHV1, 76% and 62% for OHIO, 61% and 44% for PSE-4, and 55% and 32% for the PC1 penicillinase of *S. aureus*.

In conclusion, a new Tmp^r gene is reported. The uniqueness of the new gene was confirmed by comparing the predicted protein sequence with those of types I, II, III, V, VI, VII, IX, and X and with the partial sequences of type IV and the IIIb from a nursing-home isolate of *S. sonnei*. It is also interesting to note that different strains, as defined by different plasmid patterns, showed hybridization to the probe for this new gene. On the basis of published and unpublished reports of Tmp^r genes, we tentatively classify this new resistance gene as *dhfrxii* and its product as DHFR type XII.

REFERENCES

1. **Amyes, S. G. B., and K. J. Towner.** 1990. Trimethoprim resistance; epidemiology and molecular aspects. J. Med. Microbiol. **31**:1–19.
2. **Amyes, S. G. B., K. J. Towner, G. I. Carter, C. J. Thomson, and H.-K. Young.** 1989. The type VII dihydrofolate reductase: a novel plasmid-encoded trimethoprim-resistant enzyme from gram-negative bacteria isolated in Britain. J. Antimicrob. Chemother. **24**:111–119.
3. **Barg, N. L., F. S. Hutson, L. A. Wheeler, C. J. Thomson, S. G. B. Amyes, M. Wharton, and W. Schaffner.** 1990. Novel dihydrofolate reductases isolated from epidemic strains of trimethoprim/sulfamethoxazole-resistant *Shigella sonnei*. J. Infect. Dis. **162**:466–473.
4. **Currier, T. C., and E. W. Nester.** 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. **76**:431–441.
5. **Feinberg, A., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–267.
6. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
7. **Fling, M. E., J. Kopf, and C. Richards.** 1988. Characterization of plasmid pAZ1 and the type III dihydrofolate reductase gene. Plasmid **19**:30–38.
8. **Fling, M. E., and C. Richards.** 1983. The nucleotide sequence of the trimethoprim-resistant dihydrofolate reductase gene harbored by Tn7. Nucleic Acids Res. **11**:5147–5158.
9. **Fling, M. E., L. Walton, and L. P. Elwell.** 1982. Monitoring of plasmid-encoded, trimethoprim-resistant dihydrofolate reductase genes: detection of a new resistant enzyme. Antimicrob. Agents Chemother. **22**:882–888.
10. **Goldstein, F. W., B. Papadopoulou, and J. F. Acar.** 1986. The changing pattern of trimethoprim resistance in Paris, with a review of worldwide experience. Rev. Infect. Dis. **8**:725–737.
11. **Huovinen, P.** 1987. Trimethoprim resistance. Antimicrob. Agents Chemother. **31**:1451–1456.
12. **Jansson, C., and O. Skold.** 1991. Appearance of a new trimethoprim resistance gene, *dhfrIX*, in *Escherichia coli* from swine. Antimicrob. Agents Chemother. **35**:1891–1899.
13. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
14. **Maas, R.** 1983. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid **10**:296–298.
15. **Murray, B. E., T. Alvarado, K.-H. Kim, M. Vorachit, P. Jayanetra, M. M. Levin, I. Prenzel, M. Fling, L. Elwell, G. H. McCracken, G. Madrigal, C. Odio, and L. R. Trabulsi.** 1985. Increasing resistance to trimethoprim-sulfamethoxazole among isolates of *Escherichia coli* in developing countries. J. Infect. Dis. **152**:1107–1113.
16. **Murray, B. E., and R. C. Moellering, Jr.** 1979. Aminoglycoside-modifying enzymes among clinical isolates of *Acinetobacter calcoaceticus* subsp. *anitratus* (*Herellea vaginicola*): explanation for high-level aminoglycoside resistance. Antimicrob. Agents Chemother. **15**:190–199.
17. **Murray, B. E., E. Rensimer, and H. L. DuPont.** 1982. Emergence of high level trimethoprim resistance in fecal *Escherichia coli* during oral administration of trimethoprim or trimethoprim/sulfamethoxazole. N. Engl. J. Med. **306**:130–135.
18. **Murray, B. E., and E. R. Rensimer.** 1983. Transfer of trimethoprim resistance from fecal *Escherichia coli* isolated during a prophylaxis study in Mexico. J. Infect. Dis. **147**:724–728.
19. **National Committee for Clinical Laboratory Standards.** 1985. Performance standards for antimicrobial disk susceptibility test. Publication M2-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
20. **Parsons, Y., R. M. Hall, and H. W. Stokes.** 1991. A new trimethoprim resistance gene, *dhfrX*, in the In7 integron of plasmid pDGO100. Antimicrob. Agents Chemother. **35**:2436–2439.
21. **Reves, R. R., M. Fong, L. K. Pickering, A. Bartlett III, M. Alvarez, and B. E. Murray.** 1990. Risk factors for fecal colonization with trimethoprim-resistant and multiresistant *Escherichia coli* among children in day-care centers in Houston, Texas. Antimicrob. Agents Chemother. **34**:1429–1434.
22. **Reves, R. R., B. E. Murray, L. K. Pickering, D. Prado, M. Maddock, and A. V. Bartlett III.** 1987. Children with trimethoprim- and ampicillin-resistant fecal *Escherichia coli* in day care centers. J. Infect. Dis. **156**:758–762.
23. **Smith, D. R., and J. M. Calvo.** 1980. Nucleotide sequence of the *E. coli* gene coding for dihydrofolate reductase. Nucleic Acids Res. **8**:2255–2274.
24. **Sundstrom, L., P. Radstrom, G. Swedberg, and O. Skold.** 1988. Site-specific recombination promotes linkage between trimethoprim- and sulfonamide resistance genes. Sequence characterization of *dhfrV* and *sulI* and a recombination active locus of Tn*21*. Mol. Gen. Genet. **213**:191–201.
25. **Sundstrom, L., P. Radstrom, G. Swedberg, and O. Skold.** 1990. Recombinational spread of the trimethoprim resistance gene, *dhfrVII*, borne on transposon Tn*5086*, demonstrates a general dissemination mechanism. Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 837.
26. **Sundstrom, L., T. Vinayagamoorthy, and O. Skold.** 1987. Novel type of plasmid-borne resistance to trimethoprim. Antimicrob. Agents Chemother. **31**:60–66.
27. **Thomson, C. J., N. Barg, and G. B. Amyes.** 1990. N-terminal amino acid sequence of the novel type IIIb trimethoprim-resistant plasmid-encoded dihydrofolate reductase from *Shigella sonnei*. J. Gen. Microbiol. **136**:673–677.
28. **Thomson, C. J., H.-K. Young, and S. G. B. Amyes.** 1990.

N-terminal amino-acid sequence and subunit structure of the type IV trimethoprim-resistant plasmid-encoded dihydrofolate reductase. J. Med. Microbiol. **32**:153–158.

29. **Toneguzzo, F., S. Glynn, E. Levi, S. Mjolsness, and A. Hayday.** 1988. Use of a chemically modified T7 DNA polymerase for manual and automated sequencing of supercoiled DNA. Bio-Techniques **6**:460–469.
30. **Volz, K. W., D. A. Matthews, R. A. Alden, S. T. Freer, C. Hansch, B. T. Kaufman, and J. Kraut.** 1982. Crystal structure of avian dihydrofolate reductase containing phenyltriazine and NADPH. J. Biol. Chem. **257**:2528–2536.
31. **Wanger, A. R., B. E. Murray, P. Echeverria, J. J. Mathewson, and H. L. DuPont.** 1988. Enteroinvasive *Escherichia coli* in travelers with diarrhea. J. Infect. Dis. **158**:640–642.
32. **Wells, C. L., R. P. Podzorski, P. K. Peterson, N. K. Ramsay, R. L. Simmons, and F. S. Rhame.** 1984. Incidence of trimethoprim-sulfamethoxazole-resistant enterobacteriaceae among transplant recipients. J. Infect. Dis. **150**:699–706.
33. **Wylie, B. A., and H. J. Koornhof.** 1991. Nucleotide sequence of dihydrofolate reductase type VI. J. Med. Microbiol. **35**:214–218.
34. **Young, H.-K., M. V. Jesudason, G. Koshi, and S. G. B. Amyes.** 1986. Unusual expression of new low-level-trimethoprim-resistance plasmids. J. Clin. Microbiol. **24**:61–64.