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The *dhfrI* Trimethoprim Resistance Gene of Tn*7* Can Be Found at Specific Sites in Other Genetic Surroundings

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The *dhfrI* gene, mediating high-level trimethoprim resistance, was earlier found only on Tn*7*. Evidence is given here for an alternative location of this gene at a site identical to sites observed earlier for *dhfrII* on plasmid R388, *dhfrV* on pLMO20, and *aadA* on Tn*21*. All these genes and *dhfrI* are precisely inserted as discrete GTTA-flanked elements at distinct loci in very conserved surrounding sequences. One of these *dhfrI* insertions was observed to occur in association with a similarly inserted *aadA* nucleotidyltransferase gene, which mediates streptomycin and spectinomycin resistance. Close to the insertion site, there is an open reading frame translating into a 337-amino-acid peptide which shows striking similarities to recombinases of the integrase family. *sulI*, the sulfonamide resistance gene, is very often found close to the insertion point forming a genetic surrounding, originally observed as a part of Tn*21*-like transposons. The alleged integration mechanism thus provides a recombination pathway for the genetic linkage of sulfonamide and other antibiotic resistance genes, including the most frequently encountered gene for trimethoprim resistance, *dhfrI*. Furthermore, the newly observed location of *dhfrI* could shed light on the evolution of the antibiotic resistance region of Tn*7*, which could be able to take up genes by the same mechanism as that of Tn*21*-like transposons.

Trimethoprim is an efficient and inexpensive antibacterial agent which is used the world over, often in combination with a sulfonamide. Resistance, usually plasmid borne, to the antifolate effect of trimethoprim is rather common. It is mediated by plasmid-borne genes for drug resistance variations of the target enzyme, dihydrofolate reductase (DHFR). The first identified resistance enzyme of this type was that of R plasmid R483, which was later found to harbor transposon Tn*7* (1, 29). This element carries the gene *dhfrI* mediating trimethoprim resistance and the gene *aadA* expressing resistance to streptomycin and spectinomycin (7, 9, 28). The efficient spread of this transposon, with its sometimes chromosomal location in many pathogenic strains of enteric bacteria (13, 15), was thought to be responsible for the common occurrence of high levels of resistance to trimethoprim among clinical isolates (10, 26, 31). It should be pointed out, however, that Tn*7* does not mediate resistance to the commonly used trimethoprim preparation in which this drug is combined with a sulfonamide. Another type of plasmid-borne gene, *dhfrV*, expressing an antifolate resistance DHFR 75% identical to the type I enzyme, has been described to occur on a plasmid, pLMO20, from a clinical isolate (32, 33). This gene was found to be inserted into a structure nearly identical to the region of Tn*21* surrounding the *aadA* gene. These genetic surroundings were also observed for the location of the *dhfrIIb* gene in plasmid R388 (32). In these two cases, a sulfonamide resistance gene was found in the close downstream vicinity of the trimethoprim resistance genes.

Transposons similar to Tn*21* comprise a family of mercuric resistance elements in which heterogeneity is due mainly to variation within the part which accommodates different antibiotic resistance genes (11, 14, 18). This genetic element, containing variable insertions but otherwise showing strong sequence conservation, has also been observed elsewhere in different plasmids, e.g., R388 and R46 (4, 32). In Tn*21*, the *aadA* gene for streptomycin and spectinomycin resistance was found to be inserted at a specific site, identical to those at which *dhfrV* was inserted in pLMO20 and *dhfrII* was inserted in R388 (32). It was proposed that the highly conserved region where different resistance genes were precisely inserted constitutes a site-specific recombination system (4, 17, 22, 32). This interpretation was supported by the finding of an open reading frame (ORF) close to the insertion site and translating into a peptide of 337 amino acids which shows a striking similarity to recombinases of the integrase family (23, 32). Also, repeats of GTTA were invariably found at the borders of the inserted segments at the 3′ ends of which weakly conserved inverted repeats were also observed.

In this work, the *dhfrI* gene, earlier found in Tn*7* only, was observed to occur at the specific insertion site in several cases of the above-mentioned Tn*21*-like structures. The insert containing *dhfrI* and short flanking sequences was identical to a portion of Tn*7* previously proposed to represent a recombinational element (32). This newly found location for *dhfrI* puts it into a conserved and widespread structure that frequently also harbors sulfonamide resistance. This recombined vector structure thus mediates resistance to both components of the prevalent trimethoprim preparation, which contains a sulfonamide.

MATERIALS AND METHODS

Materials. Restriction enzymes and the Klenow fragment of DNA polymerase I were from Boehringer GmbH, Mannheim, Federal Republic of Germany, or Pharmacia, Uppsala, Sweden. T4 DNA ligase and T4 RNA ligase were purchased from New England BioLabs, Inc., Beverly, Mass. Agarose (DNA grade) was from Bio-Rad Laboratories, Richmond, Calif. Trimethoprim lactate was a gift from Wellcome Research Laboratories, Beckenham, England. Dihydrofolic acid was made from folic acid by the method of Blakley (2). [α-^{32}P]dATP and [α-^{35}S]dATP were from New England Nuclear, Dreieich, Federal Republic of Germany. The mod-

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ified T7 DNA polymerase, Sequenase, and nucleotide mixes for sequencing reactions were purchased from the U.S. Biochemical Corp., Cleveland, Ohio.

Media. For susceptibility testing, Iso-Sensitest medium (Oxoid, Basingstoke, England) was used. Otherwise, culturing was done either in the rich medium LB (19) or minimal medium M9 (16). Medium components and agar were from Difco Laboratories, Detroit, Mich.

Susceptibility testing. Resistance screening was performed by the paper disk diffusion method. Paper disks were purchased from Biodisk AB, Solna, Sweden, and used according to the manufacturer's instructions.

Bacterial strains. As host for M13mp18/19 clones, *Escherichia coli* JM105 [*thi rpsL endA sbcB15 hspR4* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*Z* Δ*M15*)] was used; and for pUC18/19 clones, *E. coli* JM83 [*ara* Δ(*lac-proAB*) *rpsL* φ80*lacZ* Δ*M15*] was used (38). Derivatives of pBR322 and transconjugants were in *E. coli* C600 (F$^-$ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ$^-$), which was made Nal^r for counterselection.

Separation of DNA fragments by gel electrophoresis. Submerged 0.7 to 2.0% agarose gels or 5% acrylamide gels were used with an electrophoresis buffer (TBE) of 89 mM Tris hydrochloride (pH 8.3), 89 mM boric acid, and 2.5 mM disodium EDTA. As size markers, *Pst*I-digested bacteriophage lambda was used. DNA fragments were isolated from electrophoresis gels by the method of Öfverstedt et al. (21).

Nucleotide sequencing. The dideoxynucleotide-chain termination method of Sanger et al. was used for nucleotide sequencing (26a). Single-stranded templates for the reaction were prepared by cloning restriction fragments in M13mp18/19 (38). Sequencing primers were either specially ordered oligodeoxynucleotides of 20 to 22 nucleotides complementary to the cloned fragments or the universal 17-nucleotide primers specific for M13mp18/19. The fragment-specific primers were used as a complement to a strategy described earlier (32). The modified T7 DNA polymerase (Sequenase) was used for elongation. Reaction mixes contained either dITP or dGTP, and [α-^{35}S]dATP was used as the labeling component. Sequence analysis was done by using a VAX-8200 computer, a Graphon 250 graphic terminal, and the UWGCG software package (5).

Preparation of filters for colony hybridization. Fresh overnight cultures in Iso-Sensitest medium supplemented with 100 μg of trimethoprim per ml for resistant strains were used as inocula for the application of bacteria to 80-cm^2 nitrocellulose membranes (Millipore). The filters were adhered to Iso-Sensitest agar plates that were dried to about half of their initial volume, 2-μl samples of the cultures were spotted in regular patterns on the filters, and the plates were incubated at 37°C for 2.5 h. The alkaline treatment and baking of filters were essentially as described earlier (33).

Hybridizations. The hybridizations and stringent washes were performed mainly as described earlier by Perbal (25). All steps from prehybridization to washings were done in rotating tubes at 0.5 rpm. Hybridizations were done at 42°C in a solution of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (16) also containing 20 mM sodium phosphate (pH 6.5), filter blocking reagents, 10% dextran sulfate, and 50% formamide. For washing buffer, 2× SSC also containing 0.2% sodium dodecyl sulfate was used. Washings were for 20 min. The filters were washed twice at 25°C and then three times at 68°C. The last washing was done in 1× SSC buffer containing 2% sodium dodecyl sulfate, and the filters were then soaked in 2× SSC and blotted dry.

Probe labeling. All probe fragments were labeled by chain elongations and primed by randomized hexanucleotides, with [α-^{32}P]dCTP as the labeling component. All nonradioactive ingredients and minicolumns for purification of the labeled probes were from Pharmacia, and a protocol from this manufacturer was followed. The reaction resulted in a labeling of 25,000 to 30,000 counts per ng of DNA fragment. For each hybridization, 40 ng was used.

Probe construction. As a probe for *dhfrI*, the *Hpa*I fragment of 0.49 kilobases (kb) from Tn*7* was used (28). To reduce technical problems with hybridization background from contaminating DNA, the fragment was cloned in the *Sma*I site of pUC18 to give pLKO627. A purified preparation of pLKO627 was digested with *Kpn*I and *Bam*HI, and the digest was separated by gel electrophoresis. The 0.50-kb fragment, containing the *Hpa*I fragment with a few base pairs at each end from the pUC18 polylinker sequence, was recovered, radioactively labeled, and used as a gene-specific probe for *dhfrI*. As a second probe, the 1,287-base-pair (bp) *Bam*HI-*Hpa*I fragment from pLMO20, which had been cloned between *Bam*HI and *Sma*I in pUC19 to give pLKO26, was used. Digestion of pLKO26 with *Kpn*I and *Bam*HI gave a fragment with the size of 1,289 bp that was used as the specific probe for the integrase-like ORF, representing the Tn*21*-like recombination system (see Fig. 5). Third, as a probe for the integrase-like ORF in Tn*7*, the 1.7-kb *Ava*I-*Hpa*I fragment from pRSS021 (31) was used without further subcloning. Finally, the 1.25-kb *Eco*RI fragment from pGS150 was used as a probe for the *tnpA* region of Tn*21* (Table 1; see Fig. 5).

Accession numbers. The accession numbers we have received from the EMBL Data Library are X17477 (pLMO150, 1,854 bp), X17478 (pLMO229, 1,376 bp), and X17479 (pLMO229, 486 bp).

RESULTS

High resistance to trimethoprim, most often transferable, is fairly common among enteric bacteria. A collection of 21 trimethoprim-resistant enterobacterial isolates, mostly from Swedish clinical laboratories, showed unusual combinations of trimethoprim resistance and resistance to other antibiotics and were further analyzed for types of trimethoprim resistance determinants and their genetic surroundings. All the isolates grew in the presence of more than 1 mg of trimethoprim per ml, and 18 could transfer their resistance by conjugation into *E. coli* C600. These conjugative plasmids and their resistance markers are listed in Table 2, together with the three *E. coli* isolates with nontransferable resistance. Eight of the plasmids mediated mercuric resistance (growth at a mercuric chloride concentration of 20 μg/ml), and seven of these also mediated sulfonamide resistance. Another group of nine plasmids mediated spectinomycin resistance but not mercuric or sulfonamide resistance. The combination of trimethoprim and spectinomycin resistance without sulfonamide resistance is reminiscent of transposon Tn*7*, which carries *dhfrI* (7, 9, 28, 32). All the strains were subjected to colony hybridization analysis with a probe consisting of a 0.49-kb *Hpa*I fragment from Tn*7* and, but for a few nucleotides only, comprising the *dhfrI* gene (see reference 28 and Materials and Methods). To our surprise, all 21 strains, including those expressing sulfonamide and mercuric resistance, gave strong hybridization signals with this probe (Table 2). This finding implied that *dhfrI* could occur outside its usual context of transposon Tn*7*, which also carries the spectinomycin resistance determinant. Partially purified extracts were prepared from the 18 transcon-

TABLE 1. Plasmids used

Plasmid	Characteristic(s)[a]	Reference
pLMO20	Ap Hg Km Su Tc Tp	33
pLMO24	Ap Hg Km Su Tc Tp	34
pLMO150	Hg Km Su Tp	This study
pLMO151	Hg Km Su Tp	This study
pLMO229	Ap Sp Tp	This study
M13mp18/19		38
pUC18/19	Ap	38
pGS150	Ap	1.25-kb *Eco*RI fragment from Tn*21* (R100) in pUC19
pRSS021	Ap Tp	31
pLKO1	Ap Su Tp	32
pLKO26	Ap	This study; 1.29-kb *Bam*HI-*Hpa*I fragment from pLKO1 in pUC18
pLKO627	Ap Tp	This study; 0.49-kb *Hpa*I fragment from pRSS021 in pUC18
pLKO750	Ap Sp Tp	This study; 9-kb *Bam*HI fragment from pLMO229 in pUC19
pLKO752	Ap Sp Tp	This study; 2.19-kb *Sph*I-*Hin*dIII fragment from pLKO750 in pUC19
pLKO901	Ap Su Tp	This study; 4.02-kb *Bam*HI fragment from pLMO150 in pUC19
pLKO902	Ap Tp	This study; 1.85-kb *Sph*I-*Hin*dIII fragment from pLKO901 in pUC18
pLKO951	Ap Su Tp	This study; 4.02-kb *Bam*HI fragment from pLMO151 in pUC19
pLKO1201	Ap Su Tp	This study; 4.02-kb *Bam*HI fragment from pLMO24 in pUC19

[a] Ap, Ampicillin resistance; Hg, mercuric chloride resistance; Km, kanamycin resistance; Sp, spectinomycin resistance; Su, sulfonamide resistance; Tc, tetracycline resistance; Tp, trimethoprim resistance.

jugant strains (Table 2) and assayed for activity by a procedure described earlier (33). DHFR activities were tested for their sensitivities to trimethoprim inhibition and heat inactivation. The DHFR levels were about 20 times higher in strain C600 harboring plasmids pLMO151, pLMO154, pLMO155, pLMO24, and pLMO161 than in strains containing the other plasmids, which nonetheless showed activities that were 5 to 10 times higher than that of a plasmid-free strain C600 which expresses only chromosomal DHFR. In all transconjugant extracts, the DHFR was inhibited to 50% only at a 0.1 mM concentration of trimethoprim. This was comparable to the value found for C600(R483), which is known to express DHFR of type I from Tn*7* (9, 29). For comparison, the DHFR of type V from pLMO20 (33) was found to be more sensitive; it was inhibited to 50% at 0.007 mM trimethoprim, while DHFR activity of type II from R388 was completely insensitive to 0.1 mM trimethoprim. Also, all extracts, including that from C600(R483), lost more than 90% of their DHFR activity by incubation at 45°C for 5 min, which is characteristic of the type I enzyme, which has a dimeric structure (20, 29). The partially purified extract from C600(pLMO151) was further purified by DEAE-cellulose chromatography. At gradient elution, it behaved precisely like DHFR I from Tn*7* and was also similar to this enzyme in

TABLE 2. Properties of plasmids from trimethoprim-resistant clinical isolates

Plasmid or strain	Resistance markers[a]	Hybridization[b]			
		int-Tn*7* (probe 1)	int-Tn*21* (probe 2)	*dhfrI* (probe 3)	*tnpA* (probe 4)
pLMO150	Hg Km Su Tp	−	+	+	−
pLMO151	Hg Km Su Tp	−	+	+	−
pLMO152	Sp Tp	+	−	+	−
pLMO153	Sp Tp	+	−	+	−
pLMO154	Hg Su Tp	−	+	+	−
pLMO155	Hg Su Tp	−	+	+	−
pLMO156	Sp Tp	+	−	+	−
pLMO157	Sp Tp	+	−	+	−
pLMO158	Sp Tp	+	−	+	−
pLMO159	Sp Tp	+	−	+	−
pLMO160	Km Tp	−	+	+	−
pLMO161	Hg Km Su Tp	−	+	+	−
pLMO162	Sp Tp	+	−	+	−
pLMO163	Hg Tp	−	+	+	+
pLMO164	Cm Hg Su Tp	−	+	+	+
pLMO165	Sp Tp	+	−	+	−
pLMO24	Ap Hg Km Su Tc Tp	−	+	+	+
pLMO229	Ap Sp Tp	−	+	+	−
E. coli					
4	Ap Ni Sp Tc Tp	+	−	+	ND[c]
14	Ap Cm Sp Tc Tp	+	−	+	ND
18	Ap Cm Hg Km Ni Sp Su Tc Tp	+	+	+	ND

[a] Cm, Chloramphenicol resistance; Ni, nitrofurantoin resistance. For other definitions, see footnote to Table 1.
[b] All plasmids were harbored in *E. coli* K-12 strain C600. For a description of the probes used, see Materials and Methods and Fig. 5.
[c] ND, Not determined.

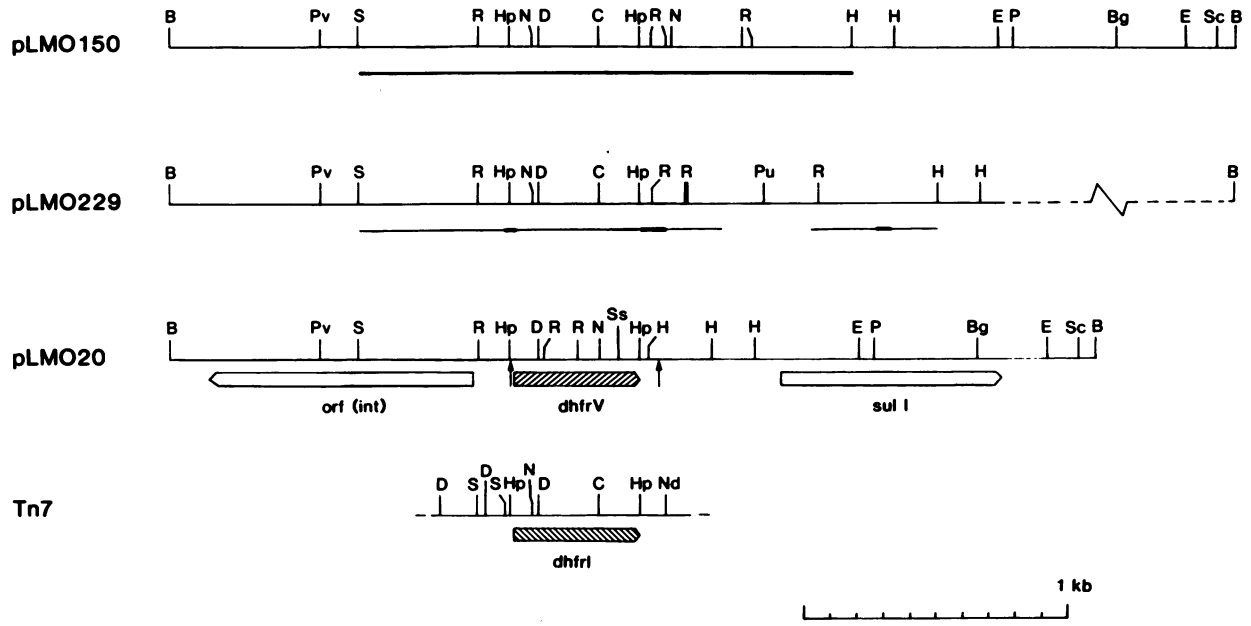

FIG. 1. Restriction maps of trimethoprim resistance fragments. Line 1, 4.0-kb *Bam*HI fragment from pLMO150, which when inserted into pUC19 gives pLKO901 of Table 1; line 2, 9-kb *Bam*HI fragment from pLMO229, which when inserted into pUC19 gives pLKO750 of Table 1; line 3, 3.5-kb *Bam*HI fragment of pLMO20 (32) (arrows mark the *dhfrV* insertion breakpoints); line 4, mapping data for the *dhfrI* area of Tn*7* (9, 28). Sequences shown in Fig. 2 to 4 are underlined with boldfaced lines, and those mentioned in the text are underlined with thin lines. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; D, *Dra*I; E, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; N, *Nco*I; Nd, *Nde*I; R, *Rsa*I; Sc, *Sac*I; S, *Sph*I; Ss, *Ssp*I; P, *Pst*I; Pu, *Pvu*I; Pv, *Pvu*II; int, integrase.

that 50% of the DHFR activity was lost when assayed in 0.07 mM trimethoprim (33).

These results thus indicated that the *dhfrI* gene also occurred outside Tn*7*. To characterize these other surroundings, DNA fragments from pLMO150 and pLMO151 containing the trimethoprim resistance determinants were cloned into the pUC19 vector. A 4.0-kb *Bam*HI fragment from each plasmid was found to mediate resistance to both trimethoprim and sulfonamide when inserted into pUC19 and transferred to host strain JM83. Despite the fact that pLMO151 expresses 20 times more DHFR than pLMO150, the restriction enzyme digestion patterns for the two fragments for *Hin*dIII, *Hpa*I, *Pst*I, *Sph*I, and *Rsa*I were identical. A map of the 4.0-kb *Bam*HI fragment from pLMO150 inserted in pUC19 to give pLKO901 is shown in Fig. 1. The characteristic locations of sites for *Bam*HI, *Pst*I, *Bgl*II, *Hin*dIII, *Eco*RV, and *Sac*I allowed the identification of the sulfonamide resistance gene as *sulI* (32). For comparison, a map representing the *sulI* area (3.5-kb *Bam*HI-*Bam*HI fragment) of pLMO20 is also shown in Fig. 1. It can be seen that the map for the pLMO150 fragment is very similar to the map for a corresponding fragment from pLMO20, with the exception of an area of about 1 kb in the middle, which in *Hpa*I, *Cla*I, *Dra*I, and *Nco*I loci resembled *dhfrI* of Tn*7* (Fig. 1). A survey of all the mapping data makes the Tn*21*-like organization of the 4.0-kb *Bam*HI fragment from pLMO150 easily recognized (32, 33). It thus seems that in this plasmid *dhfrI* has been site specifically inserted into the highly conserved Tn*21*-like structure, in much the same way as described elsewhere (32).

To study these map similarities in more detail, the nucleotide sequence was determined for the 1,854-bp *Sph*I-*Hin*dIII fragment marked out on the map of the 4.0-kb *Bam*HI fragment from pLMO150 in Fig. 1. To facilitate the M13 template constructions, this fragment was subcloned between the *Sph*I and *Hin*dIII loci of pUC18 (pLKO902 of Table 1). The sequence is shown in Fig. 2. It can be seen that there are branch points between the conserved and an inserted region of 1,084 bp with GTTA sequences at the boundaries, in analogy with earlier observations (32). The conserved region between the initial *Sph*I site and the *Hpa*I site at position 575 of Fig. 2 is identical to the pLMO20 sequence determined earlier, with only three exceptions. These are C's at positions 187 and 194 instead of G's in pLMO20 and a G at position 342 instead of a C in pLMO20 (32). Transposon Tn*21*, in this part otherwise identical to pLMO20 with the above-mentioned two exceptions (32), differs, however, from both pLMO20 and pLMO150 by a GGG insertion, which was shown to activate a second promoter for inserted genes and *sulI* by increasing the −10 to −35 distance to the optimal spacing of 17 (27, 35). The *Sph*I-*Hpa*I region contains the 5′ end of the putative site-specific recombinase and also the promoter for inserted genes (32). Following the GTTA sequence of the *Hpa*I locus is a branch point, where the similarity to Tn*21*, pLMO20, and R388 ceases. Just 18 nucleotides beyond this point an ORF starts. This is identical to the known sequence of *dhfrI* of Tn*7* (9, 28; Fig. 2). The identity with Tn*7* included the 22 nucleotides from the GTTA sequence to the GTG start codon for *dhfrI* and therefore also its ribosome-binding site. Below the DHFR gene there is a pair of inversely repeated sequences of about 44 nucleotides each, which could form an intramolecular stem-and-loop structure (Fig. 2). This genetic organization is very similar to that observed for the *dhfrV* insertion in pLMO20 (32) also in that there is a GTTA tetramer (at nucleotides 1149 to 1152 of Fig. 2), which is a sharp branch point. This is further illustrated in Fig. 3, where the pLMO150 sequence becomes identical to that of Tn*7* with the upper GTTA at the *Hpa*I locus and stays identical through the *dhfrI* gene and through the 86 nucleotides below

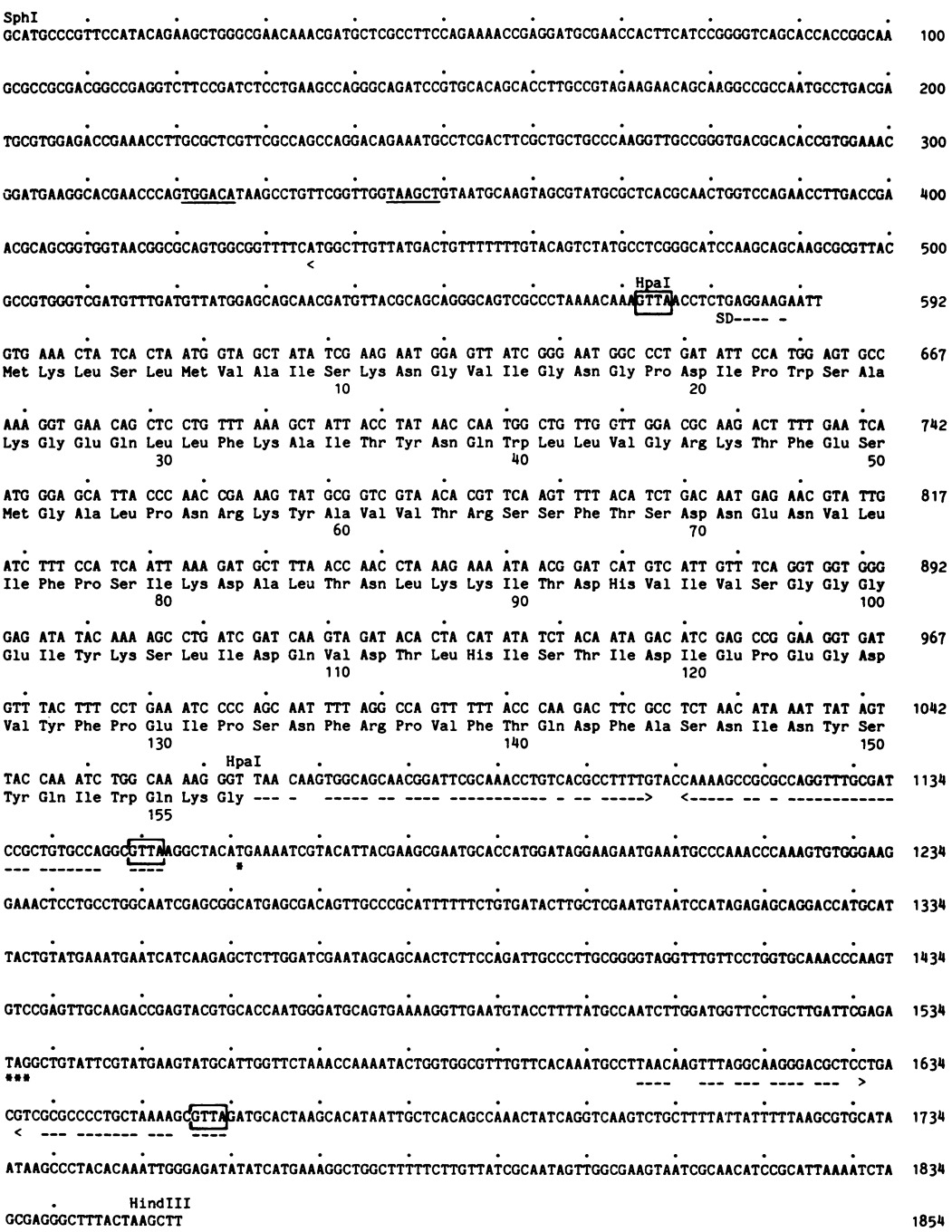

FIG. 2. Nucleotide sequence of *dhfrI* and its surroundings in pLMO150. The translated sequence of *dhfrI* comprises nucleotides 593 to 1066. The start and stop codons of the second ORF, corresponding to 125 amino acids, are marked by one and three asterisks, respectively. The start of the integrase-like ORF, reading from the opposite strand, is marked by an angle head at position 435. The promoter −35 and −10 hexamers for transcription of the *dhfrI* gene are underlined, and its translation initiation sequence is underlined and indexed by SD. The GTTA sequences at the branch points of the two inserted elements are boxed. Inverted repeat sequences, between TAAC and GTTA at the 3′ end of each inserted element, are shown by arrows.

the gene down to and including the GTTA at 1149 to 1152, after which the similarity abruptly ceases (Fig. 2 and 3). The only exception to complete identity between the 581-bp *dhfrI* elements in pLMO150 and Tn*7* was an A in the former, at nucleotide 1109 of Fig. 2, where Tn*7* has a G (9, 28). The *dhfrI* gene thus seems to be part of a cassettelike structure inserted either in Tn*7* or in the Tn*21*-like entity. Downstream from *dhfrI* and the lower GTTA there is another ORF of 125 codons, followed by an inversely repeated sequence of about 25 nucleotides and, finally, a GTTA at 1656 to 1659 (Fig. 2). After this latter GTTA the conserved sequence resumes, and there is absolute identity to the corresponding sequence of Tn*21* (32). The expression and function of the second ORF are unknown and cannot be associated with any antibiotic resistance. It thus seems that in pLMO150 there are two insertions into a Tn*21*-like structure, appearing in tandem

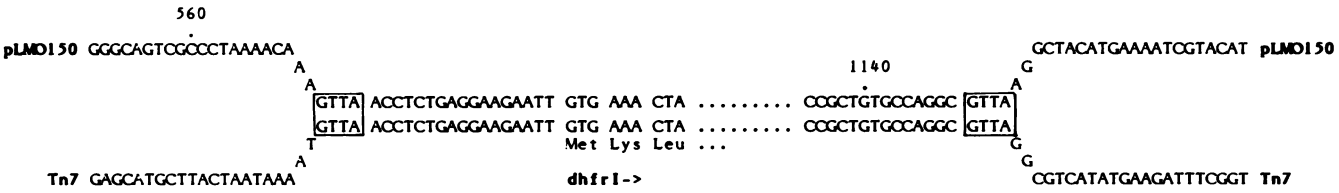

FIG. 3. Branches of inserted *dhfrI* gene element in pLMO150 and Tn*7* (28). GTTA sequences at branch points are boxed.

with one containing *dhfrI* and the other containing an unknown gene. Preliminary sequence data from the corresponding part of pLMO151 (4.0-kb *Bam*HI fragment in pLKO951; Table 1) showed identity to pLMO150, although expression of the DHFR was about 20 times higher in pLMO151 than in pLMO150. A variation in DHFR expression similar to that between pLMO150 and pLMO151 was seen for *dhfrV* in pLMO20 and pLMO28, respectively, where the resistance genes were inserted in a way very similar to those of pLMO150 and pLMO151 (33).

Another type of site-specific *dhfrI* insertion was observed in pLMO229, which mediates trimethoprim and spectinomycin resistance. In this case, *dhfrI* was inserted in tandem with an *aadA* gene, which expresses the adenylyltransferase AAD(3″) conferring resistance to streptomycin and spectinomycin. A *Bam*HI fragment of pLMO229 mediating both trimethoprim and spectinomycin resistance was ligated into the *Bam*HI locus of pUC19 to give pLKO750 (Table 1). The *Bam*HI fragment of pLKO750 had a size of 9 kb, and the restriction map of its left part as shown in Fig. 1 showed a striking similarity to the left part of the corresponding fragment from pLMO150 (Fig. 1), containing the conserved ORF for the putative site-specific recombinase mentioned above (32). The *Hpa*I, *Nco*I, *Dra*I, and *Cla*I locations indicated the presence of *dhfrI*. However, the twin *Hin*dIII sites of the pLMO150 fragment were 0.34 kb farther to the right in the pLMO229 fragment, and beyond these sites, where *sulI* is situated in pLMO150, the similarity ceased. Thus, pLMO229 resembles plasmid R751 (Tn*402*) (6, 24) in that the Tn*21*-like recombination region is present but *sulI* is not. A subclone from pLKO750 was constructed by ligating the 2.2-kb *Sph*I-*Hin*dIII fragment into pUC19 to give pLKO752 (Fig. 1; Table 1), which mediates both trimethoprim and spectinomycin resistance. The latter trait then, on the basis of similarity considerations, ought to be situated downstream of the trimethoprim resistance gene.

The *Sph*I-*Hin*dIII fragment was partially sequenced. The area from *Sph*I to the first *Hpa*I containing the promoter for inserted genes and the 5′ end of the putative recombinase was found to be identical to that of pLMO150 (Fig. 2) with one exception, at 342, where pLKO752 had a C and pLMO150 had a G. After the upper branch point at GTTA, the sequence was nearly identical to that of Tn*7* or pLMO150 and included the *dhfrI* gene and the inverted repeats mentioned above (Fig. 4). The only difference observed for the *dhfrI* gene of pLMO229 was a G at nucleotide 815 (Fig. 2) instead of a T in pLMO150 and Tn*7*. That makes codon 75 of *dhfrI* in pLMO229 translate into a valine instead of a leucine. The chromosomal enzyme from *E. coli* has a valine at the corresponding position (30). The inverted repeats just downstream from *dhfrI* in pLMO229 were identical to those of pLMO150. The similarity ceased at a GTTA tetramer, and below this an *aadA* gene, quite similar to those occurring in Tn*7* and Tn*21*, was found (7, 12). Also, the inverted repeats downstream from the *aadA* gene were identical to those seen associated with this gene in Tn*7* and Tn*21* (7, 32). Finally, the sequence beyond the lower GTTA and down to *Hin*dIII was identical to those similarly located in pLMO150, pLMO20, and Tn*21*.

In an earlier classification based on colony hybridization with a large probe representing Tn*7*, plasmid pLMO24 (Table 1) was identified as containing a trimethoprim resistance trait distinct from *dhfrI*. The trimethoprim resistance region of pLMO24 was cloned in pUC19 in a way similar to that of pLMO150 to give pLKO1201 (Table 1), which was checked by sequencing and found to be very similar if not

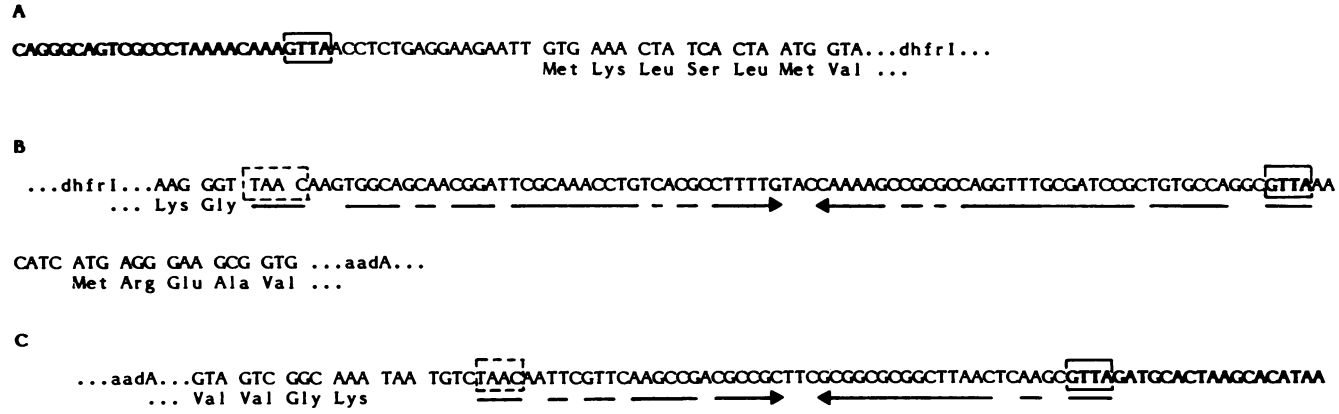

FIG. 4. Nucleotide sequences around the branch points of the tandemly integrated gene elements in pLMO229. (A) Nucleotide sequence around the 5′ end of the *dhfrI* element; (B) nucleotide sequence around the transition point between the *dhfrI* element and the *aadA* element; (C) nucleotide sequence around the lower branch point of the *aadA* element. Boldfaced letters show conserved sequences, and lightfaced letters show inserted sequences. GTTA sequences at branch points are boxed by continuous lines, and the stem-and-loop systems at the 3′ ends of the inserted elements are underlined. The TAAC sequences, inversely complementing the 3′ GTTA, are boxed by broken lines.

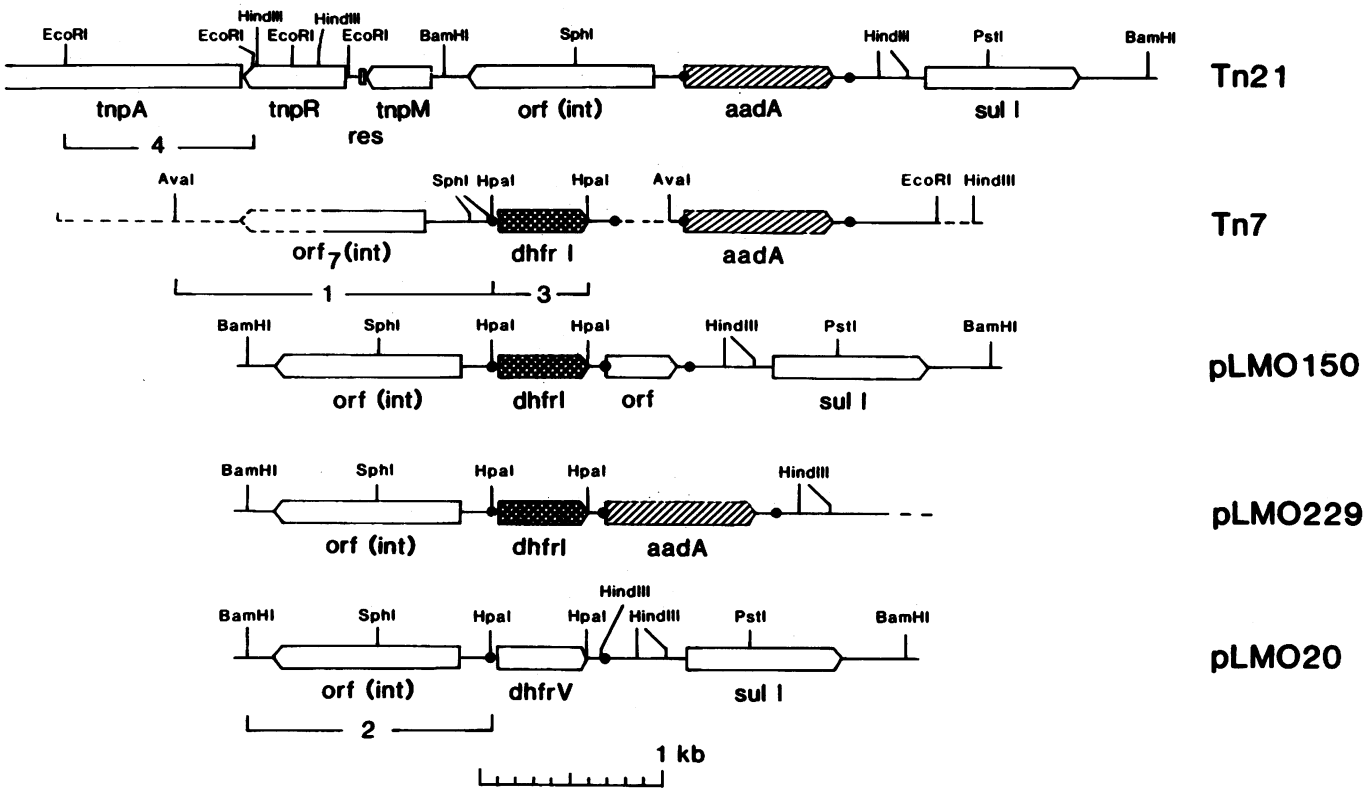

FIG. 5. Genetic organization of trimethoprim resistance gene *dhfrI* in different genetic surroundings. Parts of Tn*21* and pLMO20 (32) are shown for comparison. The fragments used as probes 1, 2, 3, and 4 are shown by brackets. Unknown sequences are shown by broken lines. Filled circles indicate cassette limits, at GTTA sequences. int, Integrase.

identical to pLKO901 derived from pLMO150 (data not shown). Thus, pLMO24 does contain *dhfrI*, and it is inserted site specifically as described above for pLMO150.

All the isolates listed in Table 2 were tested by colony hybridization using probes representing different vector structures. As mentioned above, all isolates hybridized to the *dhfrI*-specific probe (Table 2). It should be mentioned that this probe did not hybridize to strains carrying the related gene *dhfrV* (32) (not shown). Probes representing the integrase-like ORFs of Tn*7* and Tn*21*, respectively, were used as indicators of vector structures characteristic of these two transposons. Furthermore, a 1.2-kb *Eco*RI fragment containing the 5′ end of *tnpA* was used to indicate the possible presence of a complete transposable element similar to Tn*21*. A more detailed probe description is given in Materials and Methods. In Table 2 it can be seen that all 12 isolates with spectinomycin resistance, except that containing pLMO229 but including the three with nontransferable trimethoprim resistance, hybridized to the Tn*7*-specific probe. In the case of nontransferring strains, this probably indicates the occurrence of a chromosomally located Tn*7* (15). The other 10 isolates, including those containing pLMO150, pLMO151, pLMO229, and pLMO24, more fully described above, all hybridized to the probe representing the ORF typical of the Tn*21*-like structures. In general, these also mediated sulfonamide and mercuric resistance. Only three isolates, however, were found to hybridize also to the *tnpA* probe, suggesting the presence of a complete transposon similar to Tn*21*.

DISCUSSION

The very rapid spread of antibiotic resistance among pathogenic bacteria in response to the generous use of antibacterial drugs must to a large extent depend on recombinational insertion of resistance genes into plasmids and transposons. A recipient structure for the site-specific insertion of antibiotic resistance genes into Tn*21*-like transposons was described earlier (4, 11, 22, 32). This structure was in turn located on an 11.2-kb segment situated as a recombinant part on a possibly ancestral transposon, similar to Tn*501* (3), mediating mercuric resistance. In R plasmids R388 and pLMO20, the recipient site was observed to harbor the trimethoprim resistance genes *dhfrII* and *dhfrV*, respectively (32). In this work, several cases of *dhfrI* inserted into the above-mentioned recipient structure were found. The *dhfrI* gene was earlier observed in Tn*7* only. This means that the trimethoprim resistance trait in general is movable, since by now *dhfrI*, *dhfrII*, and *dhfrV* have been observed at the specific site described. Also, in the cases described here, the *dhfrI* gene was surrounded by short sequences, including inverted repeats and GTTA ends, that are identical to corresponding parts found in Tn*7*. The *dhfrI* gene and its immediate surroundings, flanked by GTTA sequences, could thus be regarded as a sort of a genetic cassette occurring in both Tn*7*- and Tn*21*-like structures (Fig. 5). This interpretation is supported by the finding of Tn*7*-like transposons, Tn*1825* and Tn*1826*, which carry different resistance genes (36). In these cases, streptothricin resistance was inserted at

a site adjacent to the *aadA* gene of Tn*7*, while the *dhfrI* gene, characteristic of Tn*7*, was missing.

Trimethoprim resistance mediated by Tn*7* is known to be very widespread among pathogenic bacteria (13, 31). The occurrence of *dhfrI* in combination with *sulI* in Tn*21*-like structures would markedly increase its potential for dissemination, both by the vector capability of the transposon and by the simultaneous spread of resistance to both components of the most widely used trimethoprim during preparation, which contains a sulfonamide.

The *aadA* genes observed here in pLMO229 and earlier in Tn*21* and Tn*7* are but for a few nucleotides identical (7, 12, 32). Since this gene is also surrounded by GTTA sequences and has inverted repeats at its 3′ end, it could be regarded as another genetic cassette inserted close to *dhfrI* in both Tn*7* and pLMO229 (Fig. 5). The occurrence of inverted repeats with the potential of forming stem-and-loop structures at the 3′ ends of the inserted genes is a constant feature of the cassette. These areas with a secondary structure potential are flanked by TAAC and GTTA at either side and comprise sequences of various lengths but with a weak conservation (4, 32, 37). In Tn*7*, the occurrence of still another potential stem-and-loop structure at the 5′ end of the *aadA* gene could represent another inserted part of Tn*7* (32). This would be a parallel to the ORF with an unknown function seen in this work to be inserted close to *dhfrI* in pLMO150 (Fig. 5). These observations indicate that the inferred cassette mechanism allows a great versatility in the recombination of different antibiotic resistance genes. The proposed interpretation is also supported by the identification of an ORF in both Tn*7* and the Tn*21*-like structures, which in translated form very much resembles site-specific recombinases of the bacteriophage integrase type (32). These predicted integraselike peptides from the ORF of Tn*7* and from that of the Tn*21*-like structure showed about 50% similarity (11, 32). Since they were sufficiently different not to cross-hybridize, probes representing these ORFs were used here to identify Tn*7* and Tn*21*-like structures among clinical isolates.

Three locations of trimethoprim resistance genes on plasmids are now known. One is the specific site on Tn*21*-like structures, mentioned here, where *dhfrI*, *dhfrII*, and *dhfrV* have now been observed. The second is Tn*7*, which also seems to have picked up *dhfrI* by a site-specific recombination mechanism. It should be mentioned here that in a recent survey of trimethoprim resistance in isolates of *Shigella* spp., 34 of 149 strains that were probe positive for *dhfrI* failed to recognize a Tn*7*-specific probe (E. Heikkilä, A. Siitonen, M. Jahkola, M. Fling, L. Sundström, and P. Huovinen, J. Infect. Dis., in press). This indicates a fairly ubiquitous spread of *dhfrI* outside Tn*7*. The third location is that of *dhfrIII* seen on a small, nonconjugative plasmid (8).

The insertion of many different antibiotic resistance genes into a conserved transposon structure resembling Tn*501*, mediating mercuric resistance, could be regarded as the evolutionary use of an old genetic structure for the purpose of counteracting the toxic attack of antibiotics. The proposed mechanism of site-specific recombination mediated by integraselike recombinases could also represent a conserved structure which originally evolved for other genetic purposes.

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