A Novel Dihydrofolate Reductase Cassette Inserted in an Integron Borne on a Tn21-Like Element

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In this study, a 498-bp *dhfrXII* gene coding for trimethoprim resistance was found inserted in a cassette-like manner in the recombinationally active locus, the integron, borne on a transposon Tn21-like element. The *dhfrXII* cassette is distinct from those cassettes earlier observed in integrons and was found here upstream of two similarly inserted cassettes. The second one carried the new unidentified *orfF*, which is 85% identical to the *orfD* cassette in R46. The third cassette contained the *aadA2* gene mediating spectinomycin resistance. The plasmid carrying this Tn21-like element was originally isolated from a trimethoprim-resistant urinary tract pathogen, *Escherichia coli*, from Turku City Hospital, Turku, Finland. By colony hybridization and polymerase chain reaction, this group of three cassettes, including *dhfrXII*, was detected in four additional *E. coli* strains of similar origin and in four *Shigella* strains isolated in Finland but originating from Asia. The dihydrofolate reductase produced from *dhfrXII* showed an unusual drug resistance in that 50% of the enzymatic activity remained at a trimethoprim concentration of 1 mM.

Trimethoprim (TMP) is an antibiotic that inhibits bacterial dihydrofolate reductase (DHFR) enzyme activity by competitively binding to the substrate binding site. Shortly after the introduction of TMP to clinical use, new plasmid-mediated DHFRs with a TMP-insensitive target site appeared among enterobacteria (1, 13, 47). A number of different plasmid-encoded DHFRs, mediating moderate- to high-level resistance to TMP, have been characterized during the last decade (26). With the exception of the DHFRs categorized as type II, all plasmid-mediated DHFRs share some degree of similarity with the chromosomal, TMP-sensitive DHFR from *Escherichia coli* (17, 29, 38, 48). DHFRs of types I, V, VI, and VII form a group of resistance enzymes with 61 to 75% amino acid sequence similarity (18, 45, 52, 55, 62). Type II DHFRs form another supergroup of DHFRs even more homologous with one another but with a structure distinct from that of any other known DHFR (7, 14, 50, 57).

The widespread *dhfrI* gene is commonly found on transposon Tn7 (5, 18, 45). Two additional antibiotic resistance genes have been found 3' of *dhfrI* in Tn7. These include *sat* coding for resistance to streptothricin (51) and *aadA1* coding for resistance to streptomycin-spectinomycin (16). All three resistance genes in Tn7 occur on mobile genetic units defined as cassettes (52-54). The cassettes are GTTPuPu-flanked elements which consist of a gene and a stem-loop structure near the 3' end (9, 21, 52, 59). Two of the consecutively inserted cassettes in Tn7 have also been found in a second defined location outside Tn7, surrounded by conserved sequences and inserted in a precise site: the *dhfrI* cassette in pLMO150, the *aadA1* gene in Tn21, and both the *dhfrI* and *aadA1* cassettes in pLMO229 (52, 54). The conserved region 5' of the specific insertion site encodes an integrase protein (3, 22, 32, 36, 37, 52) which was later demonstrated to mediate the uptake of new cassettes (10, 21, 31, 32, 51). Recently, the term integron (49) has been adopted to desig-

nate the genetic element harboring the integrase gene and the integration site (9, 36, 37, 52). Tn7 correspondingly carries a related integrase gene which, however, is supposed to be silent because of a nonsense mutation (41).

The mobile integron elements have been shown to carry many combinations of different genes for antibiotic resistance and, therefore, by their flexibility apparently to contribute to the dissemination of multiresistance (9, 36, 52). Two groups of interrelated TMP resistance genes are borne on cassettes inserted in integrons. The first of these groups includes the *dhfrI*, *dhfrV*, and *dhfrVII* genes and the second includes *dhfrII* genes (55). The mercuric resistance transposon Tn21 (19) represents one of several known locations of the integron which in Tn21 contains the *aadA1* cassette carrying the gene for adenylyltransferase (8). The integron carrying the *dhfrVII* cassette is a part of the mercuric transposon, Tn5086, closely related to Tn21 (55). Some of the integrons carrying the other TMP resistance genes disseminate via other mechanisms, like broad-host-range plasmids of Inc groups P, W, and N.

Herein, we present a new TMP resistance cassette site specifically inserted in an integron on a Tn21-like transposon. The new cassette including the *dhfrXII* gene (46) occurs in a cluster of at least two additional inserted cassettes. By its distinct structure, this gene defines a third principal type of cassettes mediating TMP resistance.

MATERIALS AND METHODS

Bacterial strains and plasmids. A urinary tract pathogen, *E. coli* TKS84, which is highly resistant to TMP and has been shown to harbor a plasmid-mediated TMP resistance mechanism, was selected for this study. The *E. coli* TKS84 strain belongs to a population of TMP-resistant *E. coli* organisms isolated from urine samples of patients at Turku City Hospital from 1987 to 1988. The isolates were previously shown to be hybridization negative with the probes for genes *dhfrI*, *dhfrIIa*, *dhfrIIb*, *dhfrIIc*, *dhfrIII*, *dhfrV*, and *dhfrVII* known to code for TMP-resistant DHFR (25).

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The *E. coli* C600 strain (4) was used as a recipient in the transformations. Plasmids pBR322 (6) and pUC19 (63) were used as cloning vectors.

Resistance determinations. MICs of TMP, co-trimoxazole, spectinomycin, streptomycin, ampicillin, and chloramphenicol were determined by the plate dilution method as described earlier (27). Resistance to sulfonamides, spectinomycin, and tetracycline was determined by the disk diffusion method (Ab Biodisk, Solna, Sweden). Iso-Sensitest agar containing 10 µg of mercuric chloride per ml was used to determine resistance to mercuric acid; for the resistance determination, *E. coli* JM105 served as a negative control.

Enzyme preparation. To study the concentration of TMP required to inhibit 50% of the enzymatic activity of DHFR, crude extracts were prepared as described by Sundström et al. (56). *E. coli* C600(pLKO632) (53) containing the *dhfrI* gene served as a control.

DNA methods. Plasmid DNA isolations, restriction enzyme digestions, and ligations were performed as described earlier (56). Transformations were carried out by the electroporation method. For sequencing, minipreparations of plasmid DNA were purified as described by Saunders and Burke (44).

DNA cloning and sequencing. Double-stranded templates of pASH1, pASH2, and pASH4 were subjected to DNA sequencing by using the dideoxy chain termination method (43) with modified T7 DNA polymerase (Sequenase version 2.0 kit; U.S. Biochemical Corp., Cleveland, Ohio) and α -³⁵S-dATP (Amersham) as a label. Primers complementary to pBR322 (pBR322E1L [5'-TACACGGTGCCTGACTGCGT] and pBR322H3R [5'-CCTGACGTCTAAGAAACCAT]) as well as primers deduced from the newly sequenced DNA were used in sequencing reactions. DNA and amino acid sequences were handled and analyzed with the Geneus (23) and Genetics Computer Group (University of Wisconsin) computer programs (11).

PCR. To further explore the expression of the region of the fragment containing the putative TMP resistance gene, polymerase chain reaction (PCR) amplification was employed. The primers used for PCR were PROM (5'-GCGAAGCTTGCCGGGTGACGCACA) and TERM (5'-GCGAAGCTTCAGTGGTGCTAACG). DNA amplification was performed as described by Saiki et al. (42). The PCR product was digested with restriction endonuclease *Hind*III and cloned into the *Hind*III site in pBR322. The recombinant plasmid, including pBR322 and the PCR product, was transferred to *E. coli* C600 by electroporation; and clones resistant to TMP were selected by plating onto TMP-containing media. Insertion of the correct fragment into pBR322 (i.e., the segment containing the open reading frame [ORF] and the upstream sequences) into the TMP-resistant clones was confirmed by another PCR, using the minipreparations of the TMP-resistant clones as templates and oligonucleotides PROM and TERM as primers. The presence of a DNA fragment of the expected size (about 800 bp) was visualized by agarose gel electrophoresis and ethidium bromide staining. A 1-kb ladder (Bethesda Research Laboratories, Gaithersburg, Md.) was used as a molecular size standard. Primers used for PCR amplification of the cassette area were complementary to sequences close to the *Sph*I site (CASS1 [5'-TGATCCGCATGCCCGTTCCATACAG]) and the *Hind*III site (CASS2 [5'-GGCAAGCTTAGTAAAGCCCTCGCTAG]).

Colony hybridization. For colony hybridization, nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) with bacterial colonies of 150 TMP-resistant *E. coli* (urinary tract pathogen) strains isolated from patients at Turku City Hos-

pital from 1987 to 1988 as well as 175 TMP-resistant *Shigella* strains isolated in Finland from 1985 to 1987 and in 1988 were prepared as described earlier (24, 25). A 22-nucleotide (nt) oligonucleotide specific for *dhfrXII* (DHFR12 [5'-GTCTATCGGCAAGCCTCTACCG]) was used as a probe. To detect the gene required for transposition in Tn21-like transposons, *tnpA* (58), a 29 nt oligonucleotide probe (PTN21TNPA [5'-CCGCCGCCGAGCGGGAAAGCCTGCTGGCG]) was employed. The probes were 5' labeled by using the 5'-DNA Terminus Labeling System with T4 kinase (Bethesda Research Laboratories). Prehybridization was carried out in a buffer consisting of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 25 mM sodium phosphate (pH 6.5), 5× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 250 µg of single-stranded salmon sperm DNA per ml for 2 h at 42°C. Hybridization was performed in the same buffer supplemented with 15% formamide (22-nt probe) or 40% formamide (29-nt probe) for 15 to 20 h at 42°C. After hybridization, the filters were washed twice for 15 min at room temperature in 2× SSC-0.1% SDS and twice for 15 min at 42°C in 0.5× SSC-0.1% SDS. Kodak X-Omat films, using intensifying screens at -70°C, were used for autoradiography.

Nucleotide sequence accession number. The nucleotide sequence data reported here have been assigned the EMBL GenBank accession number Z21672.

RESULTS

Cloning and sequencing of the determinant for TMP resistance. The urinary tract pathogen *E. coli* TKS84, which is highly resistant to TMP (MIC, 1,000 µg/ml), was shown earlier to be hybridization negative with the probes specific for the *dhfrI*, *dhfrIIa*, *dhfrIib*, *dhfrIic*, *dhfrIII*, *dhfrV*, and *dhfrVII* genes (25). By agarose gel electrophoresis, TKS84 was shown to contain several plasmids. DNA from TKS84 was transferred to *E. coli* C600 by electroporation. One of the resulting transformants was shown to be TMP resistant by plating onto TMP-containing agar plates. It contained one plasmid, pEH1, of about 70 kb, the size of which was estimated from the cumulative sizes of restriction fragments. Resistance to sulfonamides, spectinomycin, streptomycin, tetracycline, ampicillin, chloramphenicol, kanamycin, and mercuric chloride was cotransferred together with TMP resistance.

The *Hind*III fragments of pEH1 were cloned in pBR322 (Fig. 1). One clone, pASH1, contained a 3-kb fragment which conferred onto *E. coli* C600 a somewhat reduced resistance to TMP (MIC, 500 µg/ml) compared with the level induced by the parental plasmid (MIC, 1,000 µg/ml). Digestion of pASH1 with *Eco*RI and *Ava*I and religation resulted in plasmids pASH2 and pASH3, respectively (Fig. 1). Transformants of both subclones were TMP sensitive, denoting that the gene for TMP resistance or its promoter coincided with the restriction sites. In addition, the *Sph*I fragments of pEH1 were ligated to pUC19 and one of the resulting clones (pASH4) carried a 4.8-kb fragment which mediated TMP resistance. Restriction mapping of pASH4 indicated a 2.4-kb overlap with pASH1 which was supposed to contain the gene for TMP resistance. Further restriction mapping of pASH1, pASH4, and pEH1 indicated additional sites for *Eco*RI, *Hind*III, and *Sph*I similar to those in Tn5086 (55), suggesting that the TMP resistance gene could be located in an integron that furthermore could be part of a Tn21-like element.

The indicated location of the TMP resistance gene and its

FIG. 1. Strategy of cloning plasmids pASH1, pASH2, pASH3, and pASH4 (see also Fig. 4 and Materials and Methods).

surroundings were analyzed by nucleotide sequencing. A 498-bp ORF potentially encoding 165 amino acids and overlapping the *EcoRI* site was identified (Fig. 2). The start of the ORF was at ATG (positions 581 to 583, Fig. 2), and the end was at TAA (positions 1076 to 1078, Fig. 2). No other significant ORF overlapped the sites for *EcoRI* and *AvaI*. The promoter was found 231 bp upstream of the ATG start codon for this ORF (Fig. 2) (57). The ATG was not preceded by the expected ribosome-binding sequence. However, this ATG is the most likely start codon, as no alternative start codon that includes the essential parts of the sequence could be found. To ascertain whether it determines TMP resistance in pASH1, this ORF together with its putative promoter was separately cloned in pBR322 by PCR amplification. The amplification primers were located just outside the promoter and the ORF stop codon, and, after cloning into the *HindIII* site of pBR322, this ORF conferred TMP resistance to a sensitive strain. Independently, an identical sequence for a DHFR gene has recently been published and named *dhfrXII* by Singh et al. (46).

The amino acid sequence deduced from the nucleotide sequence of *dhfrXII* has been compared with those of other known DHFRs (Fig. 3) (46). With the exception of type II DHFRs, all compared DHFR enzymes could readily be aligned with the translated product of *dhfrXII*. However, the

properties of the enzyme from *dhfrXII* were shown to be distinct from those of most of the known DHFRs. The concentration of TMP required to inhibit 50% of the enzymatic activity of the type XII DHFR enzyme expressed from *E. coli* C600(pASH1) was shown to be about 1 mM (0.7 mM). The corresponding value for the type I DHFR extracted from *E. coli* C600(pLKO632) (53) was 50 μ M, which is a value similar to that determined earlier for type I DHFR (39, 47). Thus, type XII DHFR is about 10 times as resistant to inhibition by TMP as the type I DHFR enzyme and comparably as resistant as type II DHFRs. Type II DHFRs, however, have a structure completely different from those of other DHFRs (2, 39).

***dhfrXII* is located on a mobile cassette.** The sequence upstream of the DHFR gene in pEH1 consisted of sequences identical to those upstream of the type V DHFR gene in pLMO20 (52). These identical sequences are integron specific and, moreover, contain the site for the integration of resistance gene cassettes, the promoter for these cassettes, and the start of the integrase gene, *int*. Further, the corresponding sequences upstream of the DHFR genes in pLMO150 (*dhfrI* [54]), R388 (*dhfrIIb* [57]), R751 (*dhfrIIc* [14]), and Tn5086 (*dhfrVII* [55]) and the sequence upstream of the streptomycin resistance gene *aadA1* in Tn21 (52) are all virtually identical. All these resistance traits of interest

GGTTGCCGGGTGACGCACACCGTGGAACGGATGAAGGCACGAACCCAGTGGACATAAGC	331	
CTGTTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAAC	391	
CTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTTCATGGCTTGTATGACTG	451	
TTTTTTGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGGTTACGCCGTGGGTCTG	511	
ATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAACAAA	571	
GTTAGCCATATGAACCTCGGAATCAGTACGCATTTATCTCGTTGCTGCGATGGGAGCCAAT	631	
↑ M N S E S V R I Y L V A A M G A N	17	
CGGGTTATTGGCAATGGTCTTAATATCCCTGGAATAATCCGGGTGAGCAGAAGATTTTT	691	
R V I G N G P N I P W K I P G E Q K I F	37	
CGCAGACTCACTGAGGGAAGTCGTTGTATGGGGCGAAGACCTTTGAGTCTATCGGC	751	
R R L T E G K V V V M G R K T F E S I G	57	
AAGCCTCTACCGAACCGTCACACATTGGTAATCTCACGCCAAGCTAACTACCGGCCACT	811	
K P L P N R H T L V I S R Q A N Y R A T	77	
GGCTGCGTAGTTGTTTCAACGCTGTCGCACGCTATCGCTTTGGCATCCGAACCTCGGCAAT	871	
G C V V V S T L S H A I A L A S E L G N	97	
GAACTCTACGTCGCGGGCGGAGCTGAGATATACACTCTGGCACTACCTCACGCCACGGC	931	
E L Y V A G G A E I Y T L A L P H A H G	117	
GTGTTTCTATCTGAGGTACATCAAACCTTCGAGGGTGACGCCTTCTTCCCAATGCTCAAC	991	
V F L S E V H Q T F E G D A F F P M L N	137	
GAAACAGAATTCGAGCTTGTCTCAACCGAAACCATTCAGCTGTAATCCGTACACCCAC	1051	
E T E F E L V S T E T I Q A V I P Y T H	157	
TCCGTTTATGCGCGTCGAAACGGCTAACCATTCGCTCAACGGGACGCCAAAATGCTGCGC	1111	
S V Y A R R N G *	165	
ATTTTGGTTCCCTCCGCTGCGCTCCGGCTCTCGTTACGTCCAACGTTAGCACCCTGAAA	1171	
----- ↑		
CCCAGCTTTATTAGCTCATGTTTATTCAAACGGCATTAGCTTTTCAGGCGTTATTACG	1231	
TGCCTGTTTTGCCTTTTTTCCGGGCTTCGCCTGCATGGGCTGCGCAGGTTTTAGTCTTT	1291	
TTGGCCTCTAGCCCTTGCCTAGCAAGCGCAAGCAGCTATCGTTTTTGCAGTGCTGTGCCG	1351	
CCTCGGTGGCGCAGCGTTTTTTACGGTTAGCGCCCGTCGCCAAATTCAAGTTATCCGTT	1411	
TTGGCTTCTGTTTCTAACATTTTCGGTCAAGCCGACCCGATTCTGCGGTGCGTTACCTC	1471	
GCCCCGTTAGACATCATGAGGGAAGCGGTGACCATCGAAATTTGAAACCAACTATCAGAGG	1531	
↑		
TGCTAAGCGTCATTGAGCGCCATCTGGAATCAACGTTGCTGGCCGTGCATTTGTACGGCT	1591	
CCGCAGTGGA.....AAAGGCGAGATCATCAAGTCAGTTGGTAAATGATGTCTAACAA	2287	
TTCTGTTCAAGCCGACGCTACGCGCGGCGGCTTAACCTCCGGCGTTAGATGCACTAAGCACA	2347	
↑		
TAATTGCTCACAGCCAACTATCAGGTCAAGTCTGCTTTTATTATTTTAAGCGTGCATA	2407	
ATAAGCCCTACACAAATTGGGAGATATATCATGAAAGGCTGGCTTTTCTTGTATCGCA	2467	
ATAGTTGGCGAAGTAATCGCAACATCCGCATTAAATCTAGCGAGGGCTTTACTAAGCTT	2527	
→ suli		

FIG. 2. Nucleotide sequence of the *dhfrXII* and *orfF* cassettes and surroundings in pEH1. The flanks of the following spectinomycin resistance cassette *aadA2* and its transition to *qacEA1* are also given (Fig. 4). Vertical arrows mark the GTT sequence constantly occurring at the beginnings of cassettes. Numbering of nucleotides starts from the *SphI* site (not included) in the *int* gene (Fig. 4) (52). The promoter hexamers are underlined. Asterisks denote stop codons.

occur in cassettes flanked by GTTPuPu sequences and carry stem-loop components at their 3' ends (9, 52, 59). In the plasmid described here, pEH1, the branch point of identity to other integrons occurred at a GTTA (Fig. 2) just upstream of the ATG start of *dhfrXII*.

Analysis of the sequence 3' of the *dhfrXII* gene revealed two further ORFs potentially expressed in the same direc-

tion. The first of these was found to be 85% similar to the unidentified cassette ORF named D (21) characterized earlier in plasmids R1767 (35), R46 (20), and R751 (41a). In plasmids R1767 and R46, *orfD* is located downstream of the OXA-2 gene cassette, while in R751, it is downstream of *dhfrIIc*. A comparison of the ORF characterized in pEH1, now named *orfF*, and *orfD* showed that they are flanked by

1	DhfrXII	MNSESVRIYL	VAAMG.ANRV	IGNGNIPW.	KIPGEQKIFR	RLTE.....	.GKVVMGRK	TFESIG...K	PLPNRHTLVI	SRQ.ANYRA.	TGCVVV.STL	SHAIALASEL	110
	DhfrVII	MK■S■	IS■TS.E■G■	■■■■■D■	SAK■LL■K	A■TY.....	.NQWLLV■	■■D■M■	V■K■Y■A■V	■K.GISS■	NEN■L■F■PSI	FI■L■Q■EL■KI	
	DhfrIX	MASL■M	IV■VN.KTGG	■■F■EN■Q■	HE■EDL■H■K	AV■M.....	.NS■L■I■	■■■A■LP■	V■G■L■H■V■V	■K■TV■P■T■Q■N.	■D■Q■V■V■Y■	QI■V■RT■L■	
	DhfrX	MN■S■	IF■NELIT■A	F■Q■G■K■L■	Q■F■K■ED■M■Q■F■	KT■.....	.NS■■■■■■LN	■WR■LP■K■M■K	L■.G■D■FI■	■ST■TE■HE■VL	M■NN■Q■IF■K■SF	ES■F■LE■AF■RD■T	
	DhfrEco	■M■S■	I■■LA.VD■	■■■MEN■AM■	N■L■AD■L■AW■K	N■L■.....	.N■P■I■	■■■H■	■W■■■■■■	■R■	■S■P■.GT.	DDR■TW■K■SV	DE■■AC■GD■V
	Human	...■M■V■G■SL■NC	IV■VS.Q■MG	■■■K■NG■DL■	■P■PL■RN■FR■Y■Q■	■M■T■TS■VE■G	K■Q■NL■I■	■K■	■WF■■■■	■PE■KN■R	■K■G■IN■■L	■EL■KE■PP■Q■	GA■H■FL■S■RS■
													DD■L■K■TE■Q■P
111	DhfrXII	GN.....ELY	VAGGABIYTL	ALPHAH..GV	FLSEVHTFE	GDAFFPMLNE	TEF..ELVST	...ETIQAVI	PYTHSV	YARRNG....			206
	DhfrVII	TD.....H■	■S■G■G■Q■	■NS	LIEK■D■.II	H■T■T■VE■V■	■IN■K■IP■	.N■..N■FE	...Q■F■LS■N■	...N■Y■Q■I	WK■G■		
	DhfrIX	VDKPEYSQIF	■I■K■SA■EN	LAA■Y■VD■.KL	Y■TR■Q■L■NT■Q■	Q■T■EL■DL■SL■F	K■SW■.K■	■■■E	...V■PT■IT	EN■K■TL■IF■Q■I	WIN■P■ISE■E	PT■C■..	
	DhfrX	TKP.....IN	■I■V■G■LL■SE	■I■E■ST■V■YM	SS■IH■MV■K■PV■H	A■V■V■V■VEL■M	N■K■LY■SD■FK■YP	EN■IL■W■VG■DP■	DS■V■Y■SL■SID■K	F■V■PAS■LV■GV	P■ND■INT		
	DhfrEco	PE.....IM.	■I■G■RV■EQ	F■K■Q■.KL	Y■TH■IDA■EV■	■TH■D■Y■EP	DD■W■.S■S■FS	...F■HD■DA	Q■NS■HS■C■FE■I	LE■N■.....			
	Human	ELANKVDMVW	IV■■SSV■KE	■MN■PG■HL■KL	■V■TR■IM■D■	■S■T■E■ID■L	E■KY■.K■L■PE	Y■PG■VL■SD■V■QE	E■KG■IK■K■FE■	■EK■ND■.....			

FIG. 3. Comparison of translated nucleotide sequences for type XII DHFR (DhfrXII), *E. coli* chromosomal DHFR (DhfrEco) (48), type VII DHFR in pLMO27 (DhfrVII) (55), type IX DHFR in pCJ001 (DhfrIX) (29), type X DHFR in pDGO100 (DhfrX) (38), and eucaryotic DHFR (Human) (33). Amino acids identical to those characterized in type XII DHFR are marked with squares. Gaps are marked by dots. The glutamic acid in type XII DHFR at position 35 in the aligned amino acid sequence is indicated with an arrow.

GTTA on either side and form stem-loops near their 3' ends. *orfD* and *orfF* most probably represent two related but distinct cassettes which, nevertheless, have no known function. Downstream of the *orfF* cassette, we found the 5' end of still another cassette carrying the gene for spectinomycin resistance that is virtually identical to the *aadA2* gene studied earlier (60). Also, the 3' border of this cassette was studied by nucleotide sequencing. Downstream of the *aadA2* cassette the *qacEΔ1* gene (40) was found. The *qacEΔ1* gene is the truncated form of the multiresistance gene *qacE* and is found in all integrons carrying the *sulI* gene for sulfonamide resistance (40).

The TMP resistance gene in pEH1 is borne on a Tn21-like element. At the other end of the 3-kb *Hind*III fragment, a 0.5-kb region was sequenced and showed 100% identity to the *res* site in Tn21 (28) and to at least 147 bp of the *tnpR* gene (5' end [12, 28]) and to at least 126 bp of the *tnpM* gene (3' end [28]). Restriction enzyme mapping showed that the organization of the region between the *int* and *tnpR* genes in pEH1 was identical to that seen in Tn21. According to the sequencing data, it seemed obvious that the determinant for TMP resistance characterized here was inserted into a transposon Tn21-like element. In Tn21, *tnpA* (58) is located downstream of the *tnpR* gene. The region downstream of the *Hind*III in *tnpR* was not confirmed by sequencing. Rather, a colony hybridization procedure with the *tnpA*-specific oligonucleotide was employed for confirmation: positive hybridization with *E. coli* C600(pEH1) showed the presence of the *tnpA* gene in pEH1. Tn21 mediates resistance to mercuric ions, a property also expressed by pEH1. Interestingly, the sequence close to the rightmost *Sph*I site of pEH1 (Fig. 4) was found to be identical to a region 3' of *merR* in plasmid pDU1358 (34a).

Probing for *dhfrXII* and the cassette area in pEH1 in clinical bacteria. By colony hybridization with an oligonucleotide probe derived from the internal sequence of *dhfrXII*, the occurrence of the *dhfrXII* gene in 150 TMP-resistant *E. coli* (urinary tract pathogen) isolates from inpatients at Turku City Hospital from 1987 to 1988 was studied. By this method, 175 TMP-resistant *Shigella* strains isolated from 1985 to 1987 and in 1988 in Finland but originating from different parts of the world (24, 25) were also studied. Four *E. coli* strains and four *Shigella* strains hybridized with the *dhfrXII* probe. All eight isolates which hybridized with the probe specific for *dhfrXII* were previously shown to be hybridization negative with probes for the *dhfrI*, *dhfrIIa*, *dhfrIIb*, *dhfrIIc*, *dhfrIII*, *dhfrV*, and *dhfrVII* genes as well as with the probe specific for transposon Tn7 (24, 25). The *Shigella* strains included one *S. flexneri* isolate from an

immigrant coming from Vietnam; the three others were *S. sonnei* originating from Thailand. All the isolates hybridizing with the probe for *dhfrXII* were resistant to TMP, cotrimoxazole, ampicillin, spectinomycin, and chloramphenicol. With the exception of the *S. flexneri* strain, all these strains grew on antibiotic sensitivity testing plates containing 10 μg of mercuric chloride per ml, which could indicate the presence of a Tn21-like transposon. By using PCR and primers specific for the *int* and the *qacEΔ1* genes flanking the inserted cassettes in pEH1, the occurrence of this cassette area in *dhfrXII*-positive isolates was studied. By PCR, 2.5-kb bands were observed for all strains studied, indicating the presence of this combination of cassettes (*dhfrXII*, *orfF*, and *aadA2*) (Fig. 4) in these strains.

DISCUSSION

The internal part of Tn21-like transposons, the integron, is a mobile entity that has been shown to harbor most of the known TMP-resistant *dhfr* genes (8, 49, 41a, 55). These resistance genes are inserted in a cassette-like manner, flanked by GTTPuPu sequences (52). The excision and insertion of the cassettes are catalyzed by the endogenous integrase protein (31), and for the recombinations, GTTPuPu serves as a target site (32). In this article, we present a new variant of DHFR cassettes that was found inserted in a Tn21-like transposon, reflecting the ubiquitous nature as well as the recombinational activity of this element. The cassette contained the *dhfrXII* gene (46), which encodes a TMP-resistant DHFR enzyme. The ORF for *dhfrXII* starts with ATG, the most commonly used translation initiation codon in procaryotic genes. In this study, the putative promoter for the ORF was found located 231 bp upstream of the start codon, identical to that seen in pLMO20 (52). However, no typical Shine-Dalgarno sequence could be detected upstream of the ATG triplet. In the *dhfrI* and *dhfrV* genes, GTG instead of ATG serves as the initiation codon (18, 52), and, in the *dhfrVII* gene, TTG is used (55).

Downstream of the DHFR cassette, another open reading frame, *orfF*, was detected (Fig. 4). This cassette was also flanked with GTTAs, and, in the 3' end, GTTA preceded by the inverted repeat, in turn preceded by TAAC (positions 1076 to 1079, Fig. 2), the inverse complement of the 5' GTTA, was found. The *orfF* has an unknown function and is similar to the *orfD* characterized in plasmid R1767 from *Salmonella typhimurium* (35), there located downstream of the OXA-2 cassette. In the sequence presented here, the *orfF* cassette was followed by a third cassette containing a variant of the *aadA2* gene which mediates resistance to

FIG. 4. Physical and genetic map of the integron region in pEH1 carrying the *dhfrXII* cassette and reference map of the corresponding region of the Tn21-like transposon Tn5086 studied earlier (55). Thick lines indicate the sequences determined here. Cassettes are boxed. Dotted lines mark the regions of pEH1 and Tn5086 amplified by PCR with primers specific for the conserved sequences flanking the cassettes (CASS1 and CASS2; see Materials and Methods). The vertical arrow marks the location of a 4.2-kb element in Tn21 which is absent in Tn5086 and pEH1. Abbreviations: A, *Ava*I; H, *Hind*III; E, *Eco*RI; S, *Sph*I.

spectinomycin and streptomycin (60). Thus, at least three antibiotic cassettes have been inserted in the integron on pEH1. The region following the cassettes in pEH1 is conserved among integrons and contains two genes, *qacEΔ1* (115 amino acids) for resistance to disinfectants (40) and *sulI* for resistance to sulfonamides (52). The *qacEΔ1* gene is a 3'-truncated form of *qacE* that occurs in plasmid R751 (40).

The 5' end of the integrase gene identical to that characterized in pLMO20 or in other integrons can be found upstream of the *dhfrXII* cassette (37, 52). The translated protein of *int*, including 337 amino acids, mediates DNA-strand exchange at the GTTPuPu sequences flanking the cassettes (32). This product belongs to the family of integrase proteins defined in particular by the presence of a conserved DNA-reactive sequence close to the C terminus (3). A similar (50% similarity) integrase-like ORF has been characterized also in Tn7 but contains a stop codon which prevents the expression of a functional integrase protein (41). In Tn21 and related transposons, the occurrence of multivariable regions of antibiotic resistance genes can be regarded as a reflection of the maintained expression of a functional integrase.

The *dhfrXII* gene encodes a TMP-resistant DHFR enzyme of 165 amino acids and with a molecular mass of 18 kDa. This is comparable to the subunit size of the type I DHFR enzyme and of the chromosomal TMP-sensitive *E. coli* DHFR (15, 18, 48). At the amino acid level, type XII DHFR shows only moderate (33 to 38%) homology to the type I, V, VI, and VII DHFRs known to be highly resistant to the competitive inhibition by TMP. The homology to the TMP-insensitive human DHFR as well as type III DHFR conferring low-level resistance to TMP was of the same magnitude,

i.e., 34 to 35% (17, 33). Despite the relatively low proportion of amino acids identical to corresponding amino acids in other DHFRs, type XII DHFR can readily be aligned with the known procaryotic and eucaryotic DHFRs (Fig. 3) (46). Thus, type XII DHFR shows the common basic structure of procaryotic or eucaryotic DHFRs. Most amino acids known to be generally conserved among DHFRs or involved in binding of cofactor, substrate, or inhibitors were intact in type XII enzyme (34). However, a noteworthy feature of type XII DHFR is a glutamic acid at the position corresponding to Asp-27 in the *E. coli* DHFR (Fig. 3) (48). In addition, the following leucine in *E. coli* is replaced by glutamine. These changes have previously been found among the family of highly resistant DHFRs, including types I, V, VI, and VII (18, 45, 52, 55, 61, 62). On the other hand, at the phenotype level type XII DHFR showed properties distinct from those of these DHFRs. The type XII DHFR enzyme was found to be even more insensitive to inhibition by TMP and in this respect comparable to type II DHFRs or TMP-insensitive eucaryotic DHFRs (7, 14, 50, 57).

The integron containing the *dhfrXII* cassette was originally isolated in a urinary tract pathogen *E. coli*, from Turku City Hospital. As pathogens causing urinary tract infections commonly manifest the fecal flora, we wanted to study whether this integron could be found in plasmids of other enteric pathogens. In this study, we found a few *Shigella* isolates, originating from Asia, that harbored multiresistant plasmids containing this integron. The *dhfrXII* gene was found in association with the *orfF* and *aadA2* cassettes in all instances studied. Simultaneous with the *Shigella* isolation, the plasmid of the same size appeared in four *E. coli* isolates from geriatric patients in Turku City Hospital. Importantly,

the patients suffering from *Shigella* enteritis were also hospitalized there. In a day-care center in the United States, the *dhfrXII* gene alone was detected by colony hybridization in 21% of TMP-resistant fecal *E. coli* isolates (46). Whether, in the long run, this DHFR gene will prove to be significant in the dissemination of TMP resistance in Finland or in other parts of the world remains to be determined.

All the bacterial DHFRs, both those that are intrinsically sensitive to TMP as well as those that are resistant, share a considerable degree of similarity. After the introduction of TMP in the late 1960s, resistant strains harboring additional plasmid-mediated, TMP-resistant DHFRs rapidly appeared. With the exception of type II DHFRs, all these plasmid-mediated DHFRs seem to be more or less related to the known bacterial DHFRs. It is an accepted fact that antibiotic resistance produced by mutational and recombinational activities in bacteria commonly follows the introduction of a new antibiotic. The TMP resistance genes found in *E. coli* seem to be genetically stable and are subject to transfer from one bacterial species to another by conjugative plasmids. Recently, it has been shown that even mycobacteria harbor the plasmid-mediated sulfonamide resistance gene *sulI*, commonly encountered in enterobacteria on an element resembling the integron of Tn21 (30). This gives an indication that the precise origin of various TMP resistance genes, borne as cassettes in integrons, could be found in quite remote bacterial genera.

REFERENCES

1. Amyes, S. G. B., and J. T. Smith. 1974. R-factor trimethoprim resistance mechanism: an insusceptible target site. *Biochem. Biophys. Res. Commun.* **58**:412-418.
2. Amyes, S. G. B., and J. T. Smith. 1976. The purification and properties of the trimethoprim-resistant dihydrofolate reductase mediated by the R-factor, R388. *Eur. J. Biochem.* **61**:597-603.
3. Argos, P., A. Landy, K. Abremski, J. B. Egan, E. Haggard-Ljungquist, R. H. Hoess, N. M. Kahn, B. Kalionis, S. V. L. Narayana, L. S. Pierson, N. Sternberg, and J. M. Leong. 1986. The integrase-family of site-specific recombinases: regional similarities and global diversity. *EMBO J.* **5**:433-440.
4. Bachmann, B. J. 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriol. Rev.* **36**:525-557.
5. Barth, P. T., N. Datta, R. W. Hedges, and N. J. Grinter. 1976. Transposition of a deoxyribonucleic acid sequence encoding trimethoprim and streptomycin resistances from R483 to other replicons. *J. Bacteriol.* **125**:800-810.
6. Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heyneker, and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**:95-113.
7. Brisson, N., and T. Hohn. 1984. Nucleotide sequence of the dihydrofolate-reductase gene borne by the plasmid R67 and conferring methotrexate resistance. *Gene* **28**:271-275.
8. Brown, N. L., T. K. Misra, J. N. Winnie, A. Schmidt, M. Seiff, and S. Silver. 1986. The nucleotide sequence of the mercury operons of plasmid R100 and transposon Tn501: further evidence for *mer* genes which enhance the activity of the mercuric ion detoxification system. *Mol. Gen. Genet.* **202**:143-151.
9. Cameron, F. H., D. J. G. Obbink, V. P. Ackerman, and R. M. Hall. 1986. Nucleotide sequence of the AAD(2') aminoglycoside adenyltransferase determinant *aadB*. Evolutionary relationship of this region with those surrounding *aadA* in R538-1 and *dhfrII* in R388. *Nucleic Acids Res.* **14**:8625-8635.
10. Collis, C. M., and R. M. Hall. 1992. Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. *J. Bacteriol.* **174**:1574-1585.
11. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
12. Diver, W. P., J. Grinstead, D. C. Fritzinger, N. L. Brown, J. Altenbuchner, P. Rogowsky, and R. Schmitt. 1983. DNA sequences of and complementation by the *tnpR* genes of Tn21, Tn501 and Tn721. *Mol. Gen. Genet.* **191**:189-193.
13. Fleming, M. P., N. Datta, and R. N. Gruneberg. 1972. Trimethoprim resistance determinant by R factors. *Br. Med. J.* **1**:726-728.
14. Flensburg, J., and R. Steen. 1986. Nucleotide sequence analysis of the trimethoprim resistant dihydrofolate reductase encoded by R plasmid R751. *Nucleic Acids Res.* **14**:5933.
15. Fling, M. E., and L. P. Elwell. 1980. Protein expression in *Escherichia coli* minicells containing recombinant plasmids specifying trimethoprim-resistant dihydrofolate reductases. *J. Bacteriol.* **141**:779-785.
16. Fling, M. E., J. Kopf, and C. Richards. 1985. Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase. *Nucleic Acids Res.* **13**:7095-7106.
17. Fling, M. E., J. Kopf, and C. Richards. 1988. Characterization of plasmid pAZ1 and the type III dihydrofolate reductase gene. *Plasmid* **19**:30-38.
18. Fling, M. E., and C. Richards. 1983. The nucleotide sequence of the trimethoprim-resistant dihydrofolate reductase harbored by Tn7. *Nucleic Acids Res.* **11**:5147-5158.
19. Grinstead, J., F. de la Cruz, and R. Schmitt. 1990. The Tn21 subgroup of bacterial transposable elements. *Plasmid* **24**:163-189.
20. Guerineaux, F., and T. Mullineaux. 1989. Nucleotide sequence of the sulfonamide resistance gene from plasmid R46. *Nucleic Acids Res.* **17**:43-70.
21. Hall, R. M., D. E. Brookes, and H. W. Stokes. 1991. Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. *Mol. Microbiol.* **5**:1941-1959.
22. Hall, R. M., and C. Vockler. 1987. The region of the IncN plasmid R46 coding for resistance to β -lactam antibiotics, streptomycin/spectinomycin and sulfonamides is closely related to antibiotic resistance segments found in IncW plasmids and in Tn21-like transposons. *Nucleic Acids Res.* **15**:7491-7501.
23. Harr, R., P. Fällman, M. Häggström, L. Wahlström, and P. Gustafsson. 1985. GENEUS, a computer system for DNA and protein sequence analysis containing information retrieval system for EMBL data library. *Nucleic Acids Res.* **14**:273-284.
24. Heikkilä, E., A. Siitonen, M. Jähkola, M. Fling, L. Sundström, and P. Huovinen. 1990. Increase of trimethoprim resistance among *Shigella* spp., 1975-1988: analysis of resistance mechanisms. *J. Infect. Dis.* **161**:1242-1248.
25. Heikkilä, E., L. Sundström, and P. Huovinen. 1990. Trimethoprim resistance in *Escherichia coli* isolates from a geriatric unit. *Antimicrob. Agents Chemother.* **34**:2013-2015.
26. Huovinen, P. 1987. Trimethoprim resistance. *Antimicrob. Agents Chemother.* **31**:1451-1456.
27. Huovinen, P., T. Mattila, O. Kiminki, L. Pulkkinen, S. Huovinen, M. Koskela, R. Sunila, and P. Toivanen. 1985. Emergence of trimethoprim resistance in fecal flora. *Antimicrob. Agents Chemother.* **28**:354-356.
28. Hyde, D. R., and C.-P. D. Tu. 1985. *tnpM*: a novel regulatory gene that enhances Tn21 transposition and suppresses cointegrate resolution. *Cell* **42**:629-638.
29. Jansson, C., and O. Sköld. 1991. Appearance of a new trimethoprim resistance gene, *dhfrIX*, in *Escherichia coli* from swine. *Antimicrob. Agents Chemother.* **35**:1891-1899.
30. Martin, C., J. Timm, J. Rauzier, R. Gomez-Lus, J. Davies, and B. Gicquel. 1990. Transposition of an antibiotic resistance element in mycobacteria. *Nature (London)* **345**:739-743.
31. Martinez, E., and F. de la Cruz. 1988. Transposon Tn21 encodes a *recA*-independent site-specific integration system. *Mol. Gen. Genet.* **211**:320-325.
32. Martinez, E., and F. de la Cruz. 1990. Genetic elements involved in Tn21 site-specific integration, a novel mechanism for the dissemination of antibiotic resistance genes. *EMBO J.* **9**:1275-1281.
33. Masters, J. N., and G. Attardi. 1983. The nucleotide sequence of

- the cDNA coding for the human dihydrofolate reductase. *Gene* 21:59–63.
34. Matthews, D. A., J. T. Bolin, J. M. Burrige, D. J. Filman, K. W. Volz, B. T. Kaufman, C. R. Beddell, J. N. Champness, D. K. Stammers, and J. Kraut. 1985. Refined crystal structure of *Escherichia coli* and chicken liver dihydrofolate reductase containing bound trimethoprim. *J. Biol. Chem.* 260:381–391.
 - 34a. Nucifora, G., L. Chu, S. Silver, and T. K. Misra. 1989. Mercury operon regulation by the *merR* gene of the organomercurial resistance system of plasmid pDU1358. *J. Bacteriol.* 171:4241–4247.
 35. Nucken, E. J., R. B. Henschke, and F. R. J. Schmidt. 1989. Nucleotide sequence of an OXA-2 beta-lactamase gene from the R-plasmid R1767 derived plasmid pBP11 and comparison to closely related resistance determinants found in R46 and Tn2603. *J. Gen. Microbiol.* 135:761–765.
 36. Ouellette, M., L. Bissonnette, and P. H. Roy. 1987. Precise insertion of determinants into Tn21-like transposons. *Proc. Natl. Acad. Sci. USA* 84:7378–7382.
 37. Ouellette, M., and P. H. Roy. 1987. Homology of ORFs from Tn2603 and from R46 to site-specific recombinases. *Nucleic Acids Res.* 15:10055.
 38. 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.
 39. Pattishall, K. H., J. Acar, J. J. Burchall, F. W. Goldstein, and R. J. Harvey. 1977. Two distinct types of trimethoprim resistant dihydrofolate reductase specified by R-plasmids of different compatibility groups. *J. Biol. Chem.* 252:2319–2323.
 40. Paulsen, I. T., T. G. Littlejohn, P. Rådström, L. Sundström, O. Sköld, G. Swedberg, and R. A. Skurray. 1993. The 3' conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. *Antimicrob. Agents Chemother.* 37:761–768.
 41. Pelletier, A., and P. H. Roy. 1990. Bacterial transposon Tn7 codes for an integrase pseudogene. *Abstr. H-243*, p. 195. 90th Annu. Meet. Am. Soc. Microbiol. 1990. American Society for Microbiology, Washington, D.C.
 - 41a. Rådström, P., et al. Unpublished data.
 42. Saiki, R., D. Gelfand, S. Stoffel, S. Scharf, R. Higuchi, G. Horn, K. Mullis, and H. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–491.
 43. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463–5467.
 44. Saunders, S. E., and J. F. Burke. 1990. Rapid isolation of miniprep DNA for double-strand sequencing. *Nucleic Acids Res.* 18:4948.
 45. Simonsen, C. C., E. Y. Chen, and A. D. Levinson. 1983. Identification of the type I trimethoprim-resistant dihydrofolate reductase specified by the *Escherichia coli* R-plasmid R483: comparison with procaryotic and eucaryotic dihydrofolate reductases. *J. Bacteriol.* 155:1001–1008.
 46. Singh, K. V., R. R. Reves, L. K. Pickering, and B. E. Murray. 1992. Identification by DNA sequence analysis of a new plasmid-encoded trimethoprim resistance gene in fecal *Escherichia coli* isolates from children in day-care centers. *Antimicrob. Agents Chemother.* 36:1720–1726.
 47. Sköld, O., and A. Widh. 1974. A new dihydrofolate reductase with low trimethoprim sensitivity induced by an R-factor mediating high level resistance to trimethoprim. *J. Biol. Chem.* 249:4324–4325.
 48. 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.
 49. Stokes, H. W., and R. M. Hall. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol. Microbiol.* 3:1669–1683.
 50. Stone, D., and S. L. Smith. 1979. The amino acid sequence of the trimethoprim-resistant dihydrofolate reductase specified in *Escherichia coli* by R-plasmid R67. *J. Biol. Chem.* 254:10857–10861.
 51. Sundström, L., P. Rådström, O. Sköld, J. Flensburg, K. Hansson, and G. Swedberg. 1991. Program Abstr. 31th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1363.
 52. Sundström, L., P. Rådström, G. Swedberg, and O. Sköld. 1988. Site-specific recombination promotes linkage between trimethoprim and sulfonamide resistance genes. Sequence characterization of *dhfrV* and *sull* and a recombinative active locus of Tn21. *Mol. Gen. Genet.* 213:191–201.
 53. Sundström, L., P. H. Roy, and O. Sköld. 1991. Site-specific insertion of three structural gene cassettes in transposon Tn7. *J. Bacteriol.* 173:3025–3028.
 54. Sundström, L., and O. Sköld. 1990. The *dhfrI* trimethoprim resistance gene of Tn7 can be found at specific sites in other genetic surroundings. *Antimicrob. Agents Chemother.* 34:642–650.
 55. Sundström, L., G. Swedberg, and O. Sköld. 1993. Characterization of transposon Tn5086, carrying the site-specifically inserted gene *dhfrVII* mediating trimethoprim resistance. *J. Bacteriol.* 175:1796–1805.
 56. Sundström, L., T. Vinayagamoorthy, and O. Sköld. 1987. Novel type of plasmid-borne resistance to trimethoprim. *Antimicrob. Agents Chemother.* 31:60–66.
 57. Swift, G., B. J. McCarthy, and F. Heffron. 1981. DNA sequence of a plasmid-encoded dihydrofolate reductase. *Mol. Gen. Genet.* 181:441–447.
 58. Ward, E., and J. Grinstead. 1987. The nucleotide sequence of *tnpA* gene in Tn21. *Nucleic Acids Res.* 15:1799–1806.
 59. Wiedemann, B., J. F. Meyer, and M. T. Zuhlsdorf. 1987. Insertions of genes into Tn21-like transposons. *J. Antimicrob. Chemother.* 18(Suppl. C):85–92.
 60. Wohlleben, W., W. Arnold, L. Bissonnette, A. Pelletier, A. Tanguay, P. H. Roy, G. C. Gamboa, G. F. Barry, E. Aubert, J. Davies, and S. A. Kagan. 1989. On the evolution of Tn21-like multiresistance transposons: sequence analysis of the gene (*aacC1*) for gentamicin acetyltransferase-3-I(AAC(3)-I), another member of the Tn21-based expression cassette. *Mol. Gen. Genet.* 217:202–208.
 61. Wylie, B. A., S. G. B. Amyes, H.-K. Young, and H. J. Koornhof. 1988. Identification of a novel plasmid-encoded dihydrofolate reductase mediating high-level resistance to trimethoprim. *J. Antimicrob. Chemother.* 22:429–435.
 62. Wylie, B. A., and H. J. Koornhof. 1991. Nucleotide sequence of dihydrofolate reductase type VI. *J. Med. Microbiol.* 35:214–218.
 63. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103–119.