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New Gene Cassettes for Trimethoprim Resistance, *dfr13*, and Streptomycin-Spectinomycin Resistance, *aadA4*, Inserted on a Class 1 Integron

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In a previous survey of 357 trimethoprim-resistant isolates of aerobic gram-negative bacteria from commensal fecal flora, hybridization experiments showed that 25% (90 of 357) of the isolates failed to hybridize to specific oligonucleotide probes for dihydrofolate reductase types 1, 2b, 3, 5, 6, 7, 8, 9, 10, and 12. Subsequent cloning and sequencing of a plasmid-borne trimethoprim resistance gene from one of these isolates revealed a new dihydrofolate reductase gene, *dfr13*, which occurred as a cassette integrated in a site-specific manner in a class 1 integron. The gene product shared 84% amino acid identity with *dfr12* and exhibited a trimethoprim inhibition profile similar to that of *dfr12*. Gene probing experiments with an oligonucleotide probe specific for this gene showed that 12.3% (44 of 357) of the isolates which did not hybridize to probes for other dihydrofolate reductases hybridized to this probe. Immediately downstream of *dfr13*, a new cassette, an aminoglycoside resistance gene of the class AADA [ANT(3″)(9)-I], which encodes streptomycin-spectinomycin resistance, was identified. This gene shares 57% identity with the consensus *aadA1* (*ant(3″)-Ia*) and has been called *aadA4* (*ant(3″)-Id*). *The 3′ end of the aadA4* cassette was truncated by IS*26*, which was contiguous with a truncated form of Tn*3*. On the same plasmid, pUK2381, a second copy of IS*26* was associated with *sul2*, which suggests that both integrase and transposase activities have played major roles in the arrangement and dissemination of antibiotic resistance genes *dfr13*, *aadA4*, bla_{TEM-1}, and *sul2*.

Class 1 integrons are DNA elements that encode a site-specific recombinase (*intI1*) of the λ family of integrases and are capable of catalyzing the insertion and recombination of mobile DNA elements (gene cassettes) at specific sites (core ite) with a consensus sequence, GTTRRRY. The integron also provides a 5′ conserved core site, *attI1*, into which gene cassettes are preferentially inserted, and the promoters responsible for expression of the cassette-encoded genes (10, 19). Gene cassettes are identified by a 59-base element which occurs at the 3′ end of the cassette and which consists of an inverted imperfect repeat of between 50 and 150 bp which has an inverse core site at the 5′ end of the inverted repeat and a core site at the 3′ end. The insertion of a gene cassette into the *attI1* site results in the formation of a secondary site (*attC*) downstream of the cassette. The *attI1* site differs from *attC* sites in that the inverse core site and secondary structure are missing 5′ of the *attI1* core site (21). The majority of published cassettes contain genes for resistance to various antimicrobial agents including trimethoprim and aminoglycosides (41). Trimethoprim selectively inhibits the bacterial dihydrofolate reductase (DHFR), thus preventing reduction of dihydrofolate to tetrahydrofolate (11). The most common mechanism of resistance to trimethoprim in enterobacteria is the production of an additional plasmid-mediated DHFR which, unlike the chromosomal enzyme, is less sensitive to inhibition by trimethoprim (6). Sixteen trimethoprim-resistant enzymes have been identified in enterobacteria and have been characterized and grouped on the basis of their sequences. With the exception of the phylogenetic group comprising DHFR types 1, 5, 6, 7, 14, and 15 and the unusual type 2 DHFRs, the trimethoprim-resistant DHFRs remain as diverse as chromosomal prokaryotic DHFRs in general (1, 45). The genes for the majority of resistant DHFRs occur as gene cassettes that are site specifically inserted into the recombinationally active site of class 1 and class 2 integrons (28). The high incidence of trimethoprim resistance gene cassettes inserted into class 1 integrons which normally harbor *sul1* (sulfonamide resistance) at the 3′ end is most likely driven by the use of trimethoprim-sulfonamide combinations, which exert strong selection pressure for coresistance (28).

Another class of genes frequently found as cassettes in the variable region of integrons encode aminoglycoside-modifying enzymes which modify aminoglycosides by acetylation, adenylation, or phosphorylation (41, 43). The adenylating enzymes comprise a phylogenetically diverse group, of which only the classes ANT(3″) and ANT(9) are related (±30% identity) (31, 35, 43). The ANT(3″) enzymes confer streptomycin-spectinomycin resistance by adenylation of the 3″-hydroxyl position of streptomycin and the 9-hydroxyl position of spectinomycin, whereas the ANT(9) class lack 3″ activity and confers resistance only to spectinomycin (43). The most frequently encountered *ant(3″)* in enterobacteria is *aadA1*, also called (*ant(3″)-Ia*) (27, 43). Gene types for two other enzymes in this class have also been established in gram-negative bacteria: *aadA2* (*ant(3″)-Ib*), which is found on pSa in *Agrobacterium tumefaciens* (9) and which shares 82% identity with *aadA1*, and an *aadA*, which is found in *Salmonella enterica* serovar Choleraesuis and which shares 44% identity with *aadA1* (32). The genes *aadA1* and *aadA2* occur as gene cassettes and are flanked by a core sequence at the 5′ end and a 59-base element at the 3′ end (9, 17).

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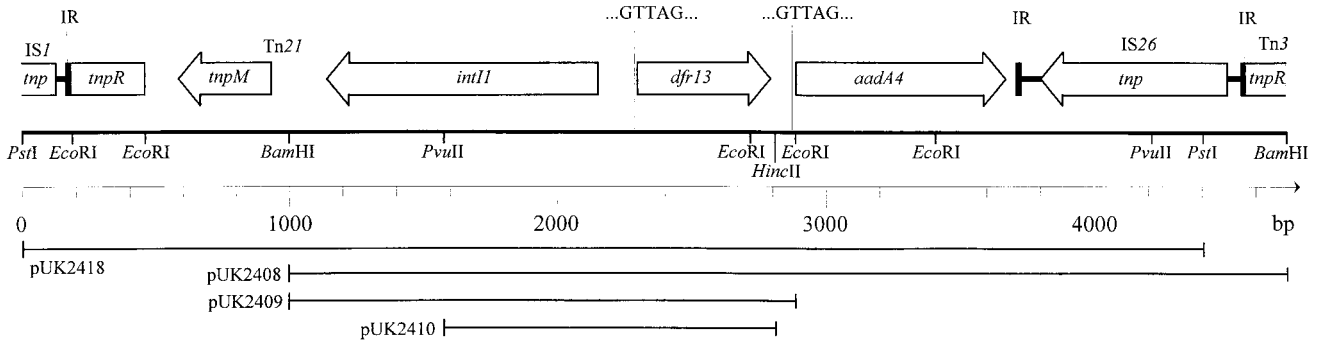

FIG. 1. Restriction map of the cloned fragments of pUK2408 and pUK2418 showing the organization and direction of transcription of *intI1*, *dfr13*, and *aadA4*, the positions of insertion sequences IS*1* and IS*26*, and the truncated sections of transposons Tn*21* and Tn*3*. The core elements of the gene cassettes and the inverted repeats (IRs) of the insertion sequence elements are indicated.

In a survey of trimethoprim resistance in South Africa, 357 isolates of gram-negative aerobic commensal fecal flora were probed with oligonucleotide probes to determine the prevalence within the population of DHFR genes that encode trimethoprim resistance (3, 4). An isolate from these studies which expressed high-level trimethoprim resistance (MIC, ≥1,024 mg/liter) and which did not hybridize to any of the DHFR probes was selected for further study. This paper reports on the molecular epidemiology, nucleotide sequence, and biochemical properties of a new DHFR gene for trimethoprim resistance and its surrounding genetic structures which include a new gene for streptomycin-spectinomycin resistance.

MATERIALS AND METHODS

Resistance determination. The MICs of trimethoprim were determined by the agar dilution method; and the antibiotic sensitivity tests for detection of resistance to tetracycline, ampicillin, chloramphenicol, spectinomycin, streptomycin, and sulfisoxazole were performed by the disk diffusion method according to the guidelines set by the National Committee for Clinical Laboratory Standards (36) as described previously (2).

Bacterial strains and plasmids. A fecal *Escherichia coli* isolate, RA33-2, was selected for this study from a group of 52 of 357 isolates which could transfer their trimethoprim resistance determinants by conjugation to an *E. coli* J62-2 recipient strain (5) and which failed to hybridize to probes for *dfr1*, *dfr2b*, *dfr3*, *dfr5*, *dfr6*, *dfr7*, *dfr8*, *dfr9*, *dfr10*, *dfr12*, and *dfr14* (3). The J62-2 transconjugant was highly resistant to trimethoprim (MIC, >2,048 mg/liter) and contained a single plasmid pUK2381 of about 70 kb, as determined by the cumulative size of *Eco*RI restriction fragments. The plasmid conferred resistance to ampicillin, spectinomycin, streptomycin, tetracycline, sulfonamides, and trimethoprim in the *E. coli* recipient. Plasmid pGEM-3Zf(+) (Promega, Madison, Wis.) was used as a cloning vector, and *E. coli* JM109 (Promega) was used as a recipient in transformation experiments. Restriction enzymes and T4 DNA ligase were supplied by Boehringer Mannheim (Mannheim, Germany) and were used with the buffers according to the manufacturer's recommendations. Purified pUK2381 DNA was restricted with *Bam*HI or *Pst*I, and the fragments were ligated into appropriately restricted pGEM-3Zf(+) DNA. The ligation mixture was electrotransformed (16) with a Gene Pulser through a Pulse Controller (Bio-Rad Laboratories, Richmond, Calif.) into *E. coli* JM109, and transformants were selected on Iso-Sensitest agar containing ampicillin (50 mg/liter) and trimethoprim (20 mg/liter). Trimethoprim-resistant transformants pUK2408 and pUK2418 contained a 3.5-kb *Bam*HI fragment and a 4.4-kb *Pst*I fragment, respectively. Plasmid pUK2408 was partially restricted with *Eco*RI, and a 5.1-kb fragment was religated and electrotransformed to produce trimethoprim-resistant clone pUK2409. The 1.9-kb *Eco*RI-*Bam*HI fragment containing the trimethoprim resistance determinant from pUK2409 was purified and was then restricted with *Hinc*II and *Pvu*II. The resulting 1,225-bp *Pvu*II-*Hinc*II fragment harbored the trimethoprim resistance determinant and was ligated into the *Sma*I site of pGEM-3Zf(+) to produce pUK2410. A restriction map of the cloned fragments is shown in Fig. 1.

Nucleotide sequencing and analysis. Double-stranded templates of pUK2408, pUK2409, pUK2418, and pUK2410 were isolated by an alkali lysis method with an additional polyethylene glycol precipitation step (7). Sequencing of alkali-denatured templates was based on the chain termination method (42), with the SEQUENASE, version 2.0, DNA sequencing kit (United States Biochemicals, Cleveland, Ohio) and a [^{35}S]dATP (Amersham, Buckinghamshire, United Kingdom) label. Sequencing was performed in both directions with both dGTP and dITP labelling mixes with M13 forward and reverse primers as well as primers deduced from the newly sequenced DNA. Sequence comparisons were performed by using the BLAST computer program (National Centre for Biological Information) and the FASTA search program (Genetics Computer Group).

DNA hybridization protocols and Southern blotting. Plasmid DNA was extracted by a modified alkali lysis procedure as described previously (2). Chromosomal DNA was isolated as described previously (7). Dot blotting and Southern hybridizations were performed with the DIG Oligonucleotide 3′-End labelling and detection kit (Boehinger Mannheim) as described previously (4). The hybridization was performed for 2 h at 53°C according to the manufacturer's recommendations. Stringency washes were performed as described previously for oligonucleotide probes (26).

Preparation and assay of DHFR. DHFR was prepared by ultrasonic disruption, followed by ammonium sulfate precipitation (50 to 80% fraction) and, finally, Sephadex G-75 gel exclusion chromatography as described previously (51). DHFR assays were performed by the method of Osborn and Huennekens (37) as described previously (6). Estimates of the protein concentrations in crude lysates were obtained by the method of Waddel (50).

Preparation and assay of adenyltransferase. Cells from logarithmic-phase cultures of *E. coli* JM109 were harvested, washed, and resuspended in 2× assay buffer (38). Cell extracts (50 μg of protein) from ultrasonically disrupted cells were assayed for adenyltransferase activity by a phosphocellulose paper binding method described previously (38). Positive and negative controls were pUK2412 (1) with *aadA1* and pGEM-3Zf(+), respectively. Estimates of the protein concentrations in crude lysates were obtained by the method of Waddel (50).

PCR. To determine the association between the novel *dfr* cassette and the class 1 integron, intragenic primers for *intI1* (5′-CGTTCCATACAGAAGCTGG-3′) and *dfr13* (5′-CCATAACGACCACTTTGCTC-3′) were used to amplify the region between the two genes. DNA amplification was performed in 50-μl volumes with 2 U of *Taq* DNA polymerase (Promega), 1 mM $MgCl_2$, and 0.1 mM deoxynucleoside triphosphates with a Hybaid (Middlesex, United Kingdom) OmniGene thermocycler on the following cycle: denaturation at 95°C for 5 min and then 30 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min.

Nucleotide sequence accession number. The nucleotide sequence data reported here have been assigned the accession no. Z50802 by EMBL.

RESULTS

Sequencing of a trimethoprim resistance cassette. Trimethoprim-resistant clone pUK2410 which contained a 1,225-bp *Pvu*II-*Hinc*II fragment was sequenced, and the first 573 nucleotides from the *Pvu*II site on the complementary strand were identical to those of the gene that encodes the first 191 amino acids of the open reading frame (ORF) of the integrase gene of class 1 integrons (*intI1*) (20, 46). The 5′ conserved sequence (5′CS) which terminates at the *attI1* core site GTTAG, which marks the point of insertion of the first gene cassette upstream from the start of *intI1*, was identical to those of class 1 integrons. Eight nucleotides downstream of the 5′CS a 498-bp ORF potentially encoding a polypeptide of 165 amino acids was identified. The start of the ORF began with an ATG start codon at positions 718 to 720 and terminated with a TAA stop codon at positions 1213 to 1215 (Fig. 2). No typical *E. coli* ribosome binding sites were identified upstream of the ATG start codon. A previously defined promoter region (33, 48) was identified within the 5′CS (underlined in Fig. 2). This has been

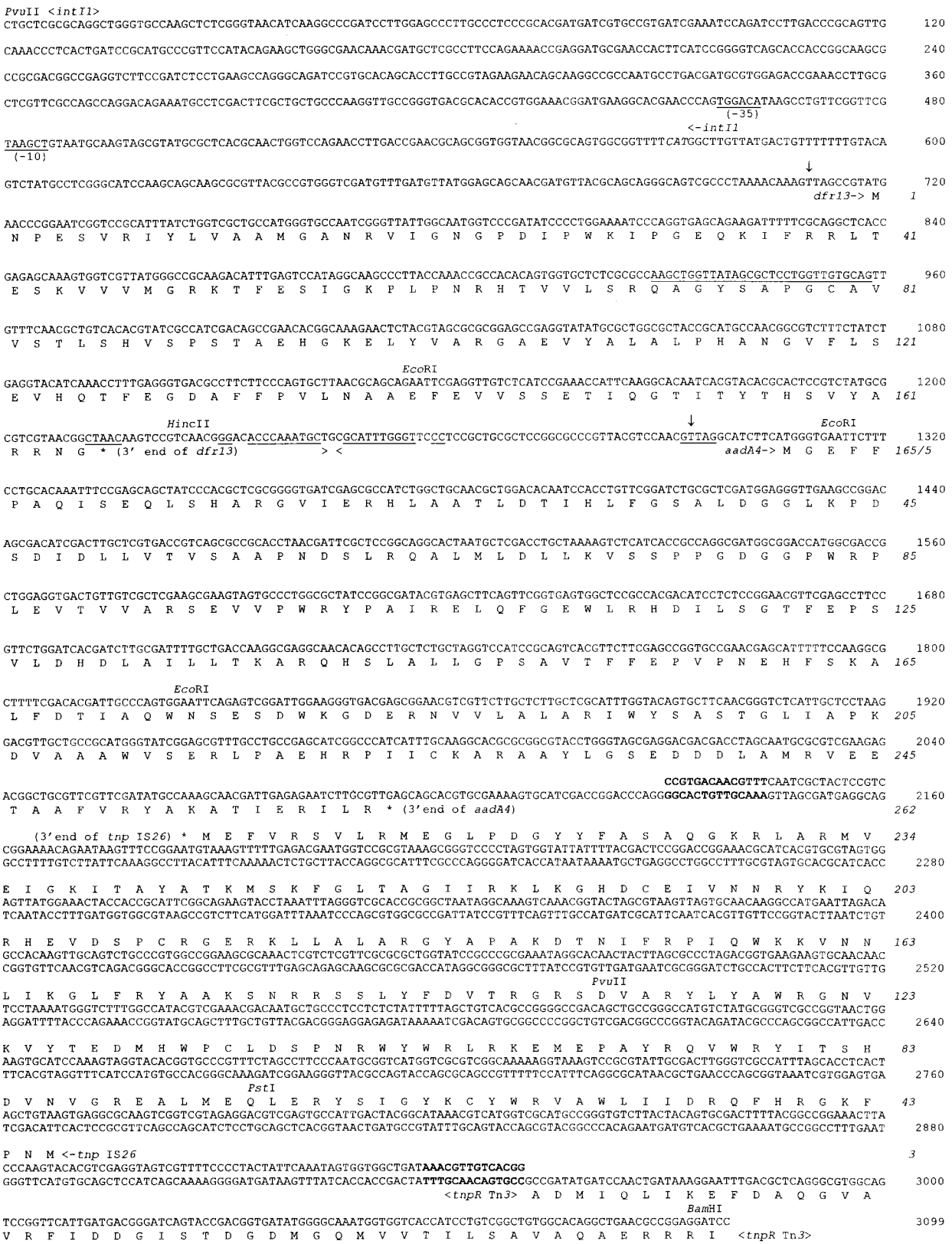

FIG. 2. Nucleotide sequence and translated peptides of the *Pvu*II-*Bam*HI fragment of pUK2381 showing the class I integron context of gene cassettes *dfr13* and *aadA4* and the position and flanking sequences of IS*26*. The −35 and −10 promoter regions of the 5′CS are underlined, the oligonucleotide probe for *dfr13* is underlined, the points of insertion of the gene cassettes are marked (↓), the imperfect inverted repeat of the 59-base element of the *dfr13* cassette is underlined and the direction is marked (><), the inverted repeats of IS*26* are in boldface type, and asterisks indicate stop codons.

TABLE 1. Comparison of adenyltransferase activities of *aadA1* and *aadA4* on different antibiotic substrates

Plasmid[a] (enzyme)	Adenylating activity with various antibiotics (cpm)[b]	
	Streptomycin	Spectinomycin
pGEM-3Zf(+) (none)	0	0
pUK2408 (*aadA4*)	4,500 ± 74	3,520 ± 190
pUK2412 (*aadA1*)	4,540 ± 140	2,420 ± 150

[a] Plasmids were expressed in *E. coli* JM109.
[b] The reported values are the average ± standard deviation for samples tested in triplicate.

experimentally defined as a weak promoter system and is identical to the promoters found within the 5′CSs of pLMO20 and Tn*5086* (46, 47). The translated polypeptide sequence was compared with other amino acid sequences in the SwissProt database. The 31 sequences with the best scores were all DHFR sequences. The amino acid sequence of the new *dfr* shared the highest degree of amino acid identity with *dfr12* (82.4%). The degrees of identity between the *dfr13* sequence and the sequences of other resistance and chromosomal DHFR genes ranged between 15 and 36%, thus placing *dfr12* and *dfr13* in an indisputable monophyletic group. The translated polypeptide for the DHFR gene is shown in Fig. 2. The novel DHFR gene has been named *dfr13*, and the encoded polypeptide has been named the type 13 DHFR. The nucleotide sequence immediately 3′ of the end of *dfr13* encoded a structure of 86 nucleotides which was recognizable as an *attC* site (59-base element) in that it began with the inverse core site CTAAC and terminated with the consensus core sequence GTTAGGC. The sequence between the two core sites differed from the sequence found between the core sites of the consensus *attC* site in that it encoded a relatively short imperfect inverted repeat which was positioned 15 bases to the left of the axis of symmetry within the element and which is similar in structure to the *dfr12* cassette (24). The *attC* site flanking *dfr13* is shown underlined in Fig. 2.

A new spectinomycin-streptomycin resistance determinant. From pUK2408 and pUK2409, the region 3′ to the DHFR gene cassette was sequenced. Twelve nucleotides downstream of the consensus core sequence (GTTAGGC) which marked the end of the *dfr13* cassette, an ORF of 262 amino acids was identified. Like many other gene cassettes, there were no recognizable promoters or ribosome binding sites between the core sequence and the first codon, and on the basis of previous experimental evidence (15), it is presumed that the promoters in the 5′CS are adequate to ensure gene expression. The amino acid sequence of the ORF shared 57.6% identity with AADA1, 54.6% identity with AADA2, and 44.3% identity with the AADA from *S. enterica* serovar Choleraesuis. The new enzyme also shared 37 and 27% identities with the AAD(9) adenyltransferases of *Staphylococcus aureus* and *Enterococcus faecalis*, respectively (31, 35). The new resistance gene had adenylating activity similar to that of *aadA1* and was 31% more efficient at adenylating spectinomycin (Table 1). The sequence identity and the 3″(9)-O-adenylating activity suggest that this enzyme is of the AADA class and has been named *aadA4* (*ant(3″)-1d*), and the translated polypeptide has been named AADA4 (ANT(3″)-Id). The MIC of spectinomycin was significantly higher for the pUK2408 clone (512 μg/ml) than for the original pUK2381 transconjugant (64 μg/ml), and this difference is most likely due to increased expression as a result of the strong promoter and the high copy number of the cloning vector. In contrast, the MIC of streptomycin for the pUK2381 transconjugant (128 μg/ml) was higher than that for the pUK2408 clone (64 μg/ml). A possible explanation for this discrepancy is the presence of an additional gene for streptomycin resistance located on pUK2381, such as *strA*, which is often associated with *sulII* (40). The pUK2381 transconjugant was sensitive to gentamicin, netilmicin, tobramycin, and amikacin.

***aadA4*, a truncated gene cassette.** A truncation due to IS*26* (34) was noticed 39 bases (nucleotides 2091 to 2130 in Fig. 2) downstream of *aadA4*. An analysis of the sequence between the 3′ end of *aadA4* and IS*26* suggests that *aadA4* is an atypical cassette, since an inverse core site YYAAC, which occurs immediately downstream of related cassettes *aadA1* and *aadA2* was absent. It is likely that *aadA4* may have originally been a functional cassette with a 59-base element located more than 39 bases downstream from the 3′ end of the ORF, prior to its interuption by the insertion of IS*26*. An analysis downstream of the IS*26* element showed that no sequences resembled a putative 59-base element and suggests that the 59-base element may have been deleted from pUK2381 through recombination with other IS*26* elements.

Insertion sequences IS*26* and IS*1*. The 3′ conserved sequence (3′CS) is usually found downstream of the variable region of class 1 integrons and contains genes that confer resistance to intercalating dyes and quaternary ammonium compounds (*qacΔE1*) and sulfonamides (*sul1*) (39). To determine whether the 3′CS was disrupted by IS*26* and is still present on pUK2381, the nucleotide sequence downstream of IS*26* was determined and was shown to be identical to the sequence of part of the resolvase gene (*tnpR*) previously identified on Tn*3* and Tn*1331* (14, 22) from amino acid 73 to amino acid 122 at the *Bam*HI site of pUK2408. Southern blotting and restriction analysis of pUK2381 with probes for *tnpR* and bla_{TEM-1} (*Sca*I-*Bgl*II fragment) suggest that *tnpR* and bla_{TEM-1} are contiguous and are arranged as determined previously for the ubiquitous element Tn*3* (14, 22). Hybridization studies of pUK2381 with gene probes consisting of the *Hin*dIII-*Pst*I fragment *sul1* and the *Hin*dIII fragment of *qacΔE1* showed that neither of these two probes hybridized to the plasmid, and therefore, the 3′CS was absent. Southern analysis with a gene probe for IS*26* revealed that two copies of the element were present on pUK2381 and suggests that this element has played a significant role in the arrangement of resistance genes in pUK2381 through transposition or cointegrate formation with IS*26*. The determinant for sulfonamide resistance was cloned into pGEM-3Zf(+). A partial sequence of this clone identified *sul2* as the sulfonamide resistance determinant. The second copy of IS*26* was located approximately 2 kb upstream of *sul2*. Partial sequencing and restriction mapping 3′ of *intI1* revealed that the integrase was contiguous with *tnpM* of Tn*21* and that the downstream *tnpR* was truncated by the insertion of IS*1*, 270 bp after the start of *tnpR* (29).

Biochemical properties of DHFR type 13. The specific activities of DHFRs from crude extracts from wild-type *E. coli* RA33-2 and the pUK2381 transconjugant were 4.0- and 2.6-fold higher than that for *E. coli* K-12, respectively. The high copy number and strong promoters of pGEM-3Zf(+) resulted in a 1,000-fold increase in the level of production of DHFR in the pUK2410 clone in comparison to that in the host chromosomal DHFR. The peak fraction of the type 13 DHFR from the Sephadex G75 column was assayed in the presence of increasing concentrations of trimethoprim and methotrexate. The trimethoprim concentration required to inhibit the activity of the type 13 DHFR by 50% (ID_{50}; ID_{50} = 800 μM) was 100,000-fold higher than that for the *E. coli* JM109 chromosomal DHFR (ID_{50} = 0.007 μM). This value is similar to that for the type 12 DHFR (ID_{50} = 700 μM) (24). The type 13

DHFR was 1,700 times more resistant to inhibition by methotrexate (ID_{50} = 5 μM) than the chromosomally encoded DHFR of *E. coli* JM109 (ID_{50} = 0.003 μM). However, the ID_{50} of methotrexate was low enough relative to those of the type 2 DHFRs (ID_{50} = 1,000 μM) to suggest that the active site for dihydrofolate (FH_2) was not dissimilar to those of other DHFR genes. From the Lineweaver-Burk plots, the Michaelis constant (K_m) for the type 13 DHFR was calculated to be 33.3 μM FH_2, which is one of the highest values for a resistant DHFR determined and which suggests that this DHFR has a 10-fold lower affinity for FH_2 than the *E. coli* chromosomal DHFR (K_m = 3.2 μM FH_2). The inhibitor constants (K_is) at trimethoprim concentrations of 250 and 500 μM were 178 and 182 μM, respectively (mean K_i = 180 μM FH_2), which is the highest K_i recorded for non-type 2 DHFRs. The duration of exposure to 45°C required to inhibit DHFR activity by 50% (TD_{50}) for the type 13 DHFR was approximately 7 min.

Molecular epidemiology of *dfr13*. On the basis of the most significant heterogeneity, a 30mer oligonucleotide probe (5′-AAGCTGGTTATAGCGCTCCTGGTTGTGCAG-3′) was derived from a comparison of the nucleotide sequences of the closely related *dfr12* and *dfr13* (nucleotides 929 to 958; Fig. 2). This heterogeneous region encodes a predicted loop between β-sheets C and D which is based on homology with the *E. coli* DHFR crystal structure (8) and overlaps the region from which the other *dfr* probes were selected (3). Of the 90 of 357 isolates of gram-negative commensal fecal flora which did not hybridize to probes for *dfr1*, *dfr2b*, *dfr3*, *dfr5*, *dfr6*, *dfr7*, *dfr8*, *dfr9*, *dfr10*, *dfr12*, and *dfr14* (3, 4), 49% (44 of 90) hybridized to the probe for *dfr13* and represented all the samples from rural and urban populations in South Africa. Fifty-eight percent (30 of 52) of self-transmissible plasmids hybridized to the probe for *dfr13*. Of the isolates which did not transfer trimethoprim resistance to recipient strain *E. coli* J62-2, 37% (14 of 38) hybridized to the probe for *dfr13*.

The *Eco*RI restriction profiles of the plasmids from the transconjugants which harbored *dfr13* showed that the majority of the plasmids exhibited unique restriction profiles; however, identical restriction fragments of the sequence which represent the resistance region were common to most of the plasmids, which suggests that a significant amount of transfer and rearrangement is associated with the element that harbors *dfr13*. The antibiograms of the plasmids that harbor *dfr13* showed that multiple drug resistance was extremely prevalent. Most of these plasmids conferred resistance to five or more antimicrobial agents. Resistance to ampicillin, tetracycline, sulfonamides, and streptomycin was virtually ubiquitous. Spectinomycin resistance was found on 23 of 30 of the plasmids, and resistance to chloramphenicol was found on 17 of 30 of the plasmids. The MICs of trimethoprim conferred by these plasmids were ≥2,048 mg/liter. To determine the association and position of *dfr13* within the integron, the region between the two genes was amplified by PCR with a primer pair whose sequence was from within the sequences of *intI1* and *dfr13*. PCR products of about 700 bp were obtained from 29 of 30 of the isolates that harbored transferable plasmids and 12 of 14 of the isolates that did not transfer resistance. The size of the PCR product suggests that the *dfr13* cassette is inserted at the *intI1* site in all these isolates.

To determine the location of *dfr13* in the isolates which did not transfer resistance, plasmid and total DNAs from the hybridization-positive isolates were run on an agarose gel, Southern blotted, and then hybridized to the *dfr13* probe. *dfr13* was located on the chromosomes of 10 of 14 of these isolates and occurred on nontransferable plasmids in the remaining 4 isolates. DNAs from isolates that harbored nontransferable trimethoprim resistance were restricted with *Bam*HI, Southern blotted, and probed with the *dfr13*-specific probe. More than half of the isolates (8 of 14) hybridized to a restriction fragment of 3.5 kb which corresponds to that of the pUK2381 control and which suggests that *intI1*, *dfr13*, *aadA4*, and the truncated version of Tn*3* are arranged on the chromosome in a manner similar to that in pUK2381. The restriction fragment sizes of the remaining hybridization-positive isolates, which included the PCR-negative isolates, were variable. The differences in the sizes of the restriction fragments indicate that different flanking sequences surround *dfr13*.

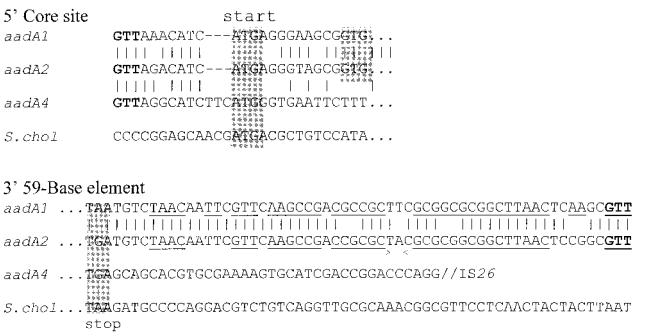

FIG. 3. Comparison of the flanking sequences of *aadA1* (17), *aadA2* (9), *aadA4*, and the *aadA* gene from *S. enterica* serovar Choleraesuis (S. chol) (32). The GTT triplet of the core sequences is marked in boldface type, and the inverted repeats of the 59-base element are underlined. Shaded areas mark the start and stop codons.

DISCUSSION

It is speculated that trimethoprim-resistant plasmid-mediated DHFRs originated as chromosomal genes that have been taken up by recombinational exchange and transferred to commensal and pathogenic organisms (45). Comparison of the *dfr12* and *dfr13* cassettes shows that flanking cassette structures of these related gene sequences share similar homologies (81%). This suggests that the genetic divergence between these closely related genes may not be the result of recent recruitment of chromosomal *dfr* genes from related organisms under selective pressure from trimethoprim but, rather, that these genes existed and diverged as gene cassettes long before the introduction of trimethoprim. Cassette homology has also been observed within other phylogenetic groups of *dfr* cassettes such as the type 1- and type 2-like classes. Similarly, a comparison of the flanking sequences of the *aadA* genes found in gram-negative bacteria (Fig. 3) shows that the cassette structures of the closely related genes *aadA1* and *aadA2* are very similar. There is no homology between the flanking sequences of the *aadA* gene of *S. enterica* serovar Choleraesuis and those of *aadA1* and *aadA2*, which suggests that *aadA* may have diverged early on from a common ancestor before *aadA1* and *aadA2* evolved as gene cassettes. In comparison, *aadA4* may be an intermediate in cassette evolution, bearing a core site at the 5′ end of the gene but completely lacking any homology to a 59-base element on what remains of the 3′ flanking sequence. It is possible that in *aadA4* a 59-base element may have been present downstream of the point of insertion IS*26*. This would explain the presence of this cassette in the context of a class 1 integron. This would imply that the structure of a putative cassette of *aadA4* is different from that of the consensus *aadA* cassette at the 3′ end.

Relative to phylogenetically related *dfr* cassettes, the class 1 integrons that harbor these cassettes are highly conserved,

which suggests that the movement of *dfr* cassettes into these elements is recent and is associated with the introduction of trimethoprim as a selective agent. The use of sulfonamides for three decades prior to the introduction of trimethoprim-sulfonamide combinations is most likely to have led to strong selection pressure for class 1 integrons with a 3′CS that harbors *sul1*. The subsequent use of trimethoprim-sulfonamide combinations has selected for the aquisition of *dfr* cassettes and the predominance of coresistance associated with the class 1 integrons (28). The arrangement of the genes for trimethoprim and sulfonamide resistance in pUK2381 is unusual, in that despite the association of *dfr13* with the 5′CS, the 3′CS of the integron which carries *sul1* is absent and has been replaced by *sul2* outside of the integron context on the same plasmid. Although *sul2* is most frequently found on small nonconjugative resistance plasmids, its presence on large plasmids has been observed previously (40). The presence of insert sequences IS*26* and IS*1* appears to have played a significant role in the evolution of pUK2381, presumably through the formation of compound transposons and/or cointegration (12). As a result, truncated forms of Tn*21* and Tn*3* have been combined to produce a more contiguous region of multiple resistance. In pUK2381, the potential for recombination by Tn*3* and Tn*21* has been substituted with insert sequences IS*26* and IS*1*, and these elements may play a role in enhancing the prevalence of this form of multiple resistance. Southern blotting and restriction analysis of isolates that harbor *dfr13* on plasmids or on the chromosome suggest that the arrangement of resistance genes, truncated transposons, and insert sequences in this element is not unique to pUK2381. *dfr13* was shown to be fairly prevalent and widespread in South Africa and was detected in 12.3% (44 of 357) of the commensal fecal isolates resistant to trimethoprim. The successful spread of this DHFR may be due to a strong selection advantage provided by the high incidence of multiple drug resistance. It is difficult to predict how widely distributed *dfr13* may be in other parts of the world. However, since this DHFR was extremely widespread in South Africa and was detected in all the rural and urban population groups, it is thought that this DHFR may be widely distributed in other parts of the world and may account for a proportion of the 10 to 68% of DHFR genes which were not identified in a number of studies (13, 23, 25, 30, 44, 49).

On the basis of the similarity between the sequence and inhibitor profile of the type 12 and 13 DHFRs, it is clear that these enzymes should be regarded as a monophyletic group (family 3) since they share similar degrees of homology to that found within the type I-like and type II-like DHFR enzyme families. Discrimination between resistant DHFR genes within close phylogenetic groups has often been hampered by the use of gene probes which are not representative of regions of adequate heterogeneity (52). The gene probing strategy used with the collection of isolates in this study (3, 4) targets gene regions which encode putative regions of low structural and functional significance. These regions are therefore most likely to be susceptible to genetic drift and therefore useful for the detection and discrimination of new gene types. This strategy has led to the detection of two new genes, *dfr15* (1) and *dfr13*, both of which share high degrees of sequence homology.

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