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Two New *dfr* Genes in Trimethoprim-Resistant Integron-Negative *Escherichia coli* Isolates▽

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Two *Escherichia coli* isolates resistant to trimethoprim but negative for integrons carried two new resistance genes, *dfrA24* and *dfrA26*, remotely similar to one another and to the cassette-independent genes *dfrA8* and *dfrA9*. The *dfrA24* gene was not associated with known mobile elements, while *dfrA26* was associated with the CR1 common region.

In previous studies of gram-negative urinary tract isolates, the prevalence of integrons was investigated. Some isolates were resistant to trimethoprim and still negative by PCR for the integrase genes of four known classes of floating integrons (classes 1 to 4) as well as for four of the *dfr* genes known at that time that were not carried as gene cassettes by integrons (*dfrA3*, *-8*, *-9*, and *-10*) (3). This finding prompted further studies of the cause of trimethoprim resistance in these isolates using a shotgun cloning approach. Two such *Escherichia coli* isolates (U21 and U93) were selected for further investigations of molecular mechanisms for trimethoprim resistance (3).

The isolates were identified at the species level according to standard methods (6). Disk diffusion tests for susceptibility were performed according to SRGA methodology using species-specific breakpoints (www.srga.org).

Shotgun cloning was performed by cleaving the extracted DNA of each isolate with one of four different restriction endonucleases, EcoRI, PstI, HindIII, or BamHI (Roche Biochemicals, Mannheim, Germany), and ligating the restriction fragments with plasmid vector pUC18 or pUC19 (Invitrogen AB, Sweden) treated with the same restriction enzyme. Ligations were transformed by heat shock into chemically competent *E. coli* TOP10 cells, a variant of strain DH10B, according to the kit protocol (Invitrogen AB, Sweden). Transformants were selected on Iso-Sensitest agar medium with 50 mg of trimethoprim/liter and 100 mg of ampicillin/liter, with only 50 mg of trimethoprim/liter, or with only 100 mg of ampicillin/liter and were incubated at 37°C for approximately 40 h.

PCR was used to generate DNA templates of cloned fragments for sequence analysis. PCR screening for the newly identified genes *dfrA24* and *dfrA26* was also performed with the original clinical sample collection. In all cases, boiled lysates of bacteria were used as the DNA template. The protocol and cycling conditions for the PCR were the same as those in previous studies, and the annealing temperatures ranged from 50 to 55°C (3).

Sequencing reactions were performed with PCR products as templates. Primers used for PCR and sequencing of cloned fragments were two forward and two reverse primers specific for the sequences upstream and downstream of the polylinker of the cloning vector and directed toward the presumed insert. The sequencing was performed using the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit, version 3.1 (Applied Biosystems), according to the protocol used previously (3).

The first trimethoprim-resistant *E. coli* isolate studied, U21, was resistant to ampicillin, trimethoprim (MIC, >32 mg/liter), cotrimoxazole, chloramphenicol, streptomycin, spectinomycin, norfloxacin, and ciprofloxacin and intermediately susceptible to cefuroxime and amoxicillin-clavulanic acid. U21 was PCR positive for the integron-connected sulfonamide resistance gene *sul1*, although it was not positive for integrons per se in any of several PCR experiments. A small HindIII fragment of 927 bp was cloned under trimethoprim selection (MIC for clone, >32 mg/liter). Nucleotide sequencing revealed the presence of an open reading frame of 558 bp, which encodes an original dihydrofolate reductase homolog and was named *dfrA24*. The DfrA24 polypeptide has 185 amino acids (aa), and among products of previous sequenced resistance genes it exhibits the highest sequence identity to the gene products of *dfrA8* (40%; 52/129 aa) and *dfrA9* (32%; 50/153 aa), neither of which is located in a gene cassette in an integron (5, 8). In comparison with all available sequences, the highest score was seen for the dihydrofolate reductase (36%; 63/175 aa) of *Porphyromonas gingivalis* (NC002950). We noticed a high amino acid sequence identity (44%; 58/126 aa) with dihydrofolate reductase enzymes in several different species of the genus *Brucella*. The sequence of the *dfrA24* gene and its immediate surroundings did not show features of integron-borne mobile gene cassettes.

The second *E. coli* isolate studied, U93, was resistant to ampicillin, sulfonamides, amoxicillin-clavulanic acid, and streptomycin in addition to its high-level resistance to trimethoprim (MIC, >32 mg/liter) but was susceptible to all other drugs tested. A 400-bp fragment with HindIII and a 1,100-bp fragment with BamHI were successfully cloned and the sequences analyzed (MICs for clones, >32 mg/liter). The inserts turned out to overlap and encoded another new *dfr*-resembling sequence. This gene was named *dfrA26* and was

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TABLE 1. Comparison of genes sharing the same breakpoint in the CR[a]

Sequence around breakpoint[b]	Following gene	Accession no.	Position
AGGTGGTTTATACTTCCTATAC/CCTAGACTCGTCTCTAGTAC	→*dfrA3b*	AY162283	5491
AGGGCGTTTATTCTTCCTATAC/GTTACTACATGACGTACTCG	→*dfrA9*	X57730	402
AGGTGGTTTATACTTCCTATAC/CCCTCGCCCTTTAAACCCTA	→*dfrA10*, $bla_{CTX-M-2}$	AJ311891	6384
AGGTGGTTTATACTTCCTATAC/CCGTAACTGCTTACAGTGAG	→*dfrA10*	L06418	5343
AGGTGGTTTATACTTCCTATAC/CCCATAAAGGAGGCATCCGG	←*qnrB2*	AM234698	5607
AGGTGGTTTATACTTCCTATAC/CCGTTAGCACCCTCCCTGAT	→*qnrA1*	AY259085	6401
AGGTGGTTTATACTTCCTATAC/CCAGTAGTGTCCCCTGTCGG	→*dfrA19*	AM234698	11326
AGGGCGTTTATTCTTCCTATAC/GTATTTGTATTCCAACCATT	→*dfrA20*	AJ605332	1098
AGGGCGTTTATTCTTCCTATAC/CCCACGATCTTGGCGTACGT	←*dfrA23*	AJ746361	5782
AGGGCGTTTATTCTTCCTATAC/GTCATTGAATCCTGATGTGA	→*dfrA26*	This work	170

[a] Including the new *dfrA26* gene identified in this work. The arrows indicate in which direction each gene is inserted. Accession numbers and positions for the breakpoint are given for each sequence.
[b] The breakpoint is indicated by a slash.

distantly related to *dfrA3* (40% identity; 72/178 aa). The gene product was related to several housekeeping dihydrofolate reductases from a range of organisms such as *Brucella suis* (AE014291) (44% identity; 75/169 aa) and *Bartonella quintana* strain Toulouse (BX897700) (48% identity; 79/164 aa). The genetic context of the *dfrA26* gene did not indicate any carriage as an integron-borne gene cassette.

However, by contrast to the *dfrA24* gene, *dfrA26* was found to be associated with common region 1 (CR1), an IS*91*-related element usually found downstream of the 3′ conserved segment region of some class 1 integrons (1, 2, 7, 9. The cloned fragment also included a short segment of a thymidylate synthase gene that was possibly linked to the dihydrofolate reductase gene in the source organism. The insertion site for the *dfrA26* downstream CR1 is identical with the breakpoints for several of the genes that have been identified in the same context, as shown in Table 1. A broad range of different resistance genes that are generally not carried as gene cassettes have been described in connection with CR1 in recent years. Examples of genes that are linked with such conserved regions and that also share the 5′ breakpoint with *dfrA26* are *dfrA3b*, *-9*, *-10*, and *-18*/*19*, *dfrA20*, and *dfrA23*. Other examples of genes in these constant regions with a capturing function are $bla_{CTX-M-9}$ (AM040708), bla_{CMY-11} (AF357599), *qnrA* (AY259085), *qnrA4* (DQ058662), *qnrB1* (DQ351241), *qnrB2* (AY931017), and *catA2* (L06822) (7, 9).

It is widely appreciated that integrons constitute a highly efficient mechanism for spreading antibiotic resistance determinants. However, neither of the two new *dfr* genes reported in this work is carried as an integron gene cassette. Scrutiny of the 19 *dfr* genes borne on cassettes (*dfrA1*, *dfrA5*, *dfrA6*, *dfrA7*, *dfrA12*, *dfrA13*, *dfrA14*, *dfrA15*, *dfrA16*, *dfrA17*, *dfrA21*, *dfrA22*, *dfrA25*, *dfrB1*, *dfrB2*, *dfrB3*, *dfrB4*, *dfrB5*, and *dfrB6*) reveals that they belong to three rather conserved classes of structural genes, and remarkably, no genes that are members of these classes have been found outside of integrons (3, 4). Our compilation of *dfr* genes reveals that, in addition to integron casette-borne genes, another large group of six *dfr* genes (*dfrA3b*, *dfrA9*, *dfrA10*, *dfrA19*, *dfrA20*, and *dfrA23*), and possibly also the gene identified in the present study, *dfrA26*, are associated with the CR. This means that there are now only four *dfr* genes established in the host by other mechanisms (*dfrA3*, *dfrA4*, *dfrA8*, *dfrA24*), including the new *dfrA24* gene, but the verification of this outgroup awaits further context information. From this assembly of data we can conclude that trimethoprim resistance in gram-negative bacteria is spread via two parallel and very efficient recombinational paths: the vast majority of the 30 trimethoprim resistance genes have been captured via one of these two mechanisms. The paucity of spreading mechanisms for the numerous types of trimethoprim resistance genes is remarkable, although it still remains possible that physical grouping of some resistance genes occurs outside of these two mechanisms. The newly identified gene in these studies, *dfrA24*, could be such a rare exception from the consensus pattern and may be spread along yet unidentified recombinational trajectories (9). Though we are still awaiting further context data, it can be preliminarily stated that *dfrA24* is linked neither with integrons nor with common regions or any other recombinatory and transposable element presently known.

Nucleotide sequence accession numbers. EMBL accession numbers for the new sequences described are AJ972619 (*dfrA24*) and AM403715 (*dfrA26*).

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