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Integron-Associated DfrB4, a Previously Uncharacterized Member of the Trimethoprim-Resistant Dihydrofolate Reductase B Family, Is a Clinically Identified Emergent Source of Antibiotic Resistance

Jacynthe L. Toulouse,[a,b,c] Thaddeus J. Edens,[d] Lorea Alejaldre,[a,b,c] Amee R. Manges,[e] Joelle N. Pelletier[a,b,c,f]

Département de biochimie, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montréal, Québec, Canada[a]; CGCC, Center in Green Chemistry and Catalysis, Montréal, Québec, Canada[b]; PROTEO, Québec Network for Protein Function, Engineering and Applications, Québec, Québec, Canada[c]; Devil's Staircase Consulting, North Vancouver, British Columbia, Canada[d]; School of Population and Public Health, University of British Columbia, Vancouver, British Columbia, Canada[e]; Département de chimie, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montréal, Québec, Canada[f]

ABSTRACT Whole-genome sequencing of trimethoprim-resistant *Escherichia coli* clinical isolates identified a member of the trimethoprim-resistant type II dihydrofolate reductase gene family (*dfrB*). The *dfrB4* gene was located within a class I integron flanked by multiple resistance genes. This arrangement was previously reported in a 130.6-kb multiresistance plasmid. The DfrB4 protein conferred a >2,000-fold increased trimethoprim resistance on overexpression in *E. coli*. Our results are consistent with the finding that *dfrB4* contributes to clinical trimethoprim resistance.

KEYWORDS type II dihydrofolate reductase, trimethoprim resistance, *E. coli* clinical isolates, *dfrB4*, antibiotic-resistant genes, class I integron, urinary tract infection

Public health agencies worldwide rank trimethoprim (TMP) a broad-spectrum antibiotic of importance in human medicine (1). Widely used as a result of its low cost and effectiveness, TMP inhibits the activity of many microbial chromosomal dihydrofolate reductases (DHFRs); thus, DHFRs have long served as prioritized targets of antiproliferative drugs (2). Although the majority of living cells harbor a chromosomal member of the type I DHFR family, encoded by a *dfrA* homolog, the *dfrB* genes encode a family of plasmid-borne type II DHFRs that are evolutionarily unrelated to type I DHFRs. The *dfrB* genes have been found in pathogenic bacteria recovered from many food sources, including fish (3), pigs (4, 5), and cows (6), where they confer TMP resistance. Bacteria carrying *dfrB* genes have also been identified in wastewater samples (7). Over the past decade, *dfrB* genes have been tracked indirectly in antibiotic resistance studies through identification of integron-related elements (8–10). Therefore, the importance of *dfrB* genes in TMP resistance in human pathogens may be underappreciated (11, 12).

To date, only seven members of the *dfrB* gene family are known, and they are highly homologous (77% to 94% genetic identity, 77% to 99% amino acid identity) (Table 1). Among these, the DfrB1 protein (also known as R67 DHFR) is the best-studied type II DHFR (13–17). It is proposed to be recently evolved, and it confers a significant survival advantage under TMP exposure to microbes that harbor it (18). To date, the family of *dfrB* genes has consistently been reported to be contained within the following mobile

Received 16 December 2016 **Returned for modification** 26 December 2016 **Accepted** 11 February 2017

Accepted manuscript posted online 27 February 2017

Citation Toulouse JL, Edens TJ, Alejaldre L, Manges AR, Pelletier JN. 2017. Integron-associated DfrB4, a previously uncharacterized member of the trimethoprim-resistant dihydrofolate reductase B family, is a clinically identified emergent source of antibiotic resistance. Antimicrob Agents Chemother 61:e02665-16. https://doi.org/10.1128/AAC.02665-16.

Address correspondence to Joelle N. Pelletier, joelle.pelletier@umontreal.ca.

TABLE 1 DNA sequence query coverage and identity of the main type II DHFRs, with the expected value

DHFR	Query coverage (% identity) and expected value[a] for:						
	dfrB1	*dfrB2*	*dfrB3*	*dfrB4*	*dfrB5*	*dfrB6*	*dfrB7*
Truncated *dfrB1*	100	95 (77) **2e−47**	90 (88) **3e−77**	89 (83) **5e−61**	100 (89) **9e−90**	100 (92) **7e−98**	100 (92) **7e−98**
dfrB2		100	85 (86) **2e−66**	72 (85) **2e−54**	83 (79) **3e−45**	97 (78) **4e−50**	95 (77) **2e−47**
dfrB3			100	94 (85) **2e−73**	96 (86) **4e−75**	97 (87) **7e−79**	96 (86) **8e−78**
dfrB4				100	92 (81) **8e−59**	94 (80) **1e−57**	92 (80) **4e−56**
dfrB5					100	100 (91) **3e−96**	100 (92) **1e−100**
dfrB6						100	93 (94) **7e−111**

[a]Expected values indicated by boldface (coding sequence only). The expected value (e) represents the probability of randomly matching two different sequences. The lower the e value, the more significant the match.

genetic elements: a highly variable 57- to 141-bp recombination binding site (*attC*) and the 7-base canonical sequence (7-be), which is identical in all *dfrB* genes (5′-GTTRRRY) except *dfrB2* (GTTAGGC) and includes a 7-be at the 3′ end, which is the reverse complement of the 7-be found upstream of the coding sequence (r7-be).

Clinical sample library. We examined whole-genome shotgun sequencing data for 593 *Escherichia coli* isolates, including 380 *E. coli* isolates recovered from 324 individuals with hospital-associated human extraintestinal infections (19) and 189 *E. coli* isolates recovered from 189 women diagnosed with a community-acquired urinary tract infection (UTI) (A. R. Manges, unpublished data). Genomic *E. coli* DNA from women with UTIs (Manges collection) was extracted using the PureLink genomic DNA minikit (Thermo Fisher Scientific). Purified DNA was sheared in water using the Biorupter Pico (Diagenode), and sequencing libraries were prepared using the TruSeq DNA PCR-free library preparation kit (Illumina), according to the manufacturer's instructions. All *E. coli* isolates were sequenced on the Illumina HiSeq 2500 at the University of British Columbia's Pharmaceutical Sciences Sequencing Centre and British Columbia Genome Sciences Centre (Vancouver, BC).

***In silico* screening.** Seven members of the *dfrB* gene family were used as templates for *in silico* screening, including two variants of the *dfrB1* gene that differ by the absence or presence of 19 additional *N*-terminal amino acids not essential for the reductase activity (20; see http://www.esi.umontreal.ca/~pelletjo/ToulouseSupplemental-material.pdf). The *in silico dfrB* screening was performed by aligning paired-end reads from 569 whole-genome-sequencing data sets to the set of *dfrB* genes in FASTA format with the Burrows-Wheeler aligner (BWA), using standard alignment parameters. Sequencing reads from *E. coli* isolates recovered from blood samples of an individual taken 2 days apart aligned exactly over the entire *dfrB4* gene sequence (19 [https://www.ncbi.nlm.nih.gov/sra/SRX560289 and https://www.ncbi.nlm.nih.gov/sra/SRX560290]). The DNA consensus sequences were identical to the previously deposited 237-bp *dfrB4* sequence (GenBank AY968808 and KP314737.1). Three further samples held one read that overlapped with the *dfrB4* integron with 0 to 2 mismatches. Because no contig within assemblies of these three data sets aligned with *dfrB4*, they were not considered further.

Reconstruction of *dfrB4* mobile genetic element and contiguous DNA segments. Read sets with significant alignment to any reference *dfrB* gene were assembled into contigs, and the *dfrB* genes were aligned with BWA to these assembled contigs. The contigs from both samples have an r7-be (GTTGGGC) 61 bp upstream of the *dfrB4* coding sequence and an *attC* sequence downstream (Fig. 1). The 7-be found in the *attC* (GCCCAAC) is 53 bp downstream from the coding sequence. These flanking sequences are identical to those of a previously reported *dfrB4* mobile genetic element (GenBank accession no. AY970968.1) from *Klebsiella pneumoniae* (21). The *attC* sequence differs by one nucleotide from another *dfrB4* mobile genetic element from *E. coli* (GenBank accession no. KP314737.1) (22; http://www.esi.umontreal.ca/~pelletjo/ToulouseSupplemental-material.pdf).

Contiguous to the mobile genetic element, we identified a 8,963-bp segment derived from sample SRX560290 and a 9,029-bp segment derived from sample

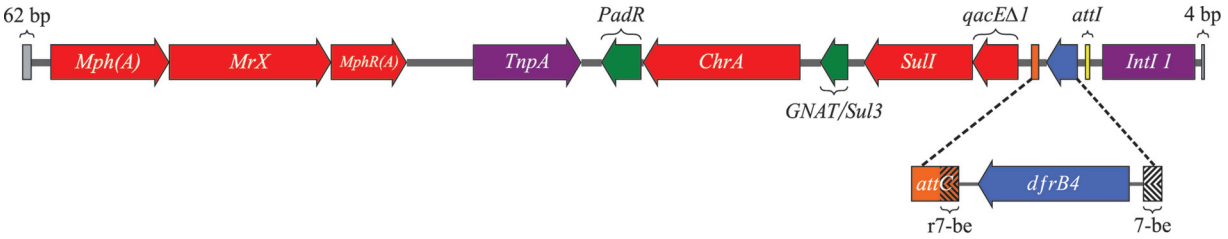

FIG 1 Scheme of the 9,029-bp DNA segment derived from sample SRX560289. Purple, indispensable genes for integration of mobile genetic elements. Yellow, *intI* (integrase/recombinase), *tnpA* (transposase), and the integron-associated recombination site (*attI*). Red, resistance genes (*qacEΔ1*, biocide resistance; *sulI*, sulfonamide resistance; the *mphR(A)/mphA/mrx* gene cluster, erythromycin resistance; and *chrA*, chromate resistance), except *dfrB4* (dihydrofolate reductase, TMP resistance), which is blue. Orange, recombination binding site (*attC*). Dashed lines, 7-be sequences. Green, DNA sequences sharing similarity with known genes: *GNAT/sul3* (acetyltransferase/sulfonamide resistance) and *padR* (transcriptional regulator). Gray, the 62- and 4-bp sequence differences between DNA contigs.

SRX560289 (Fig. 1) (19). The 66-bp difference did not belong to an open reading frame (ORF) and lay outside the *dfrB4* mobile genetic element. The *dfrB4* gene was carried with *intI1* (class 1 integron integrase/recombinase) and *tnpA* (transposase), which are indispensable for integration of mobile genetic elements (23). Moreover, multiple antibiotic, biocide, and metal resistance genes were identified: *qacEΔ1* (biocide resistance [24, 25]), *sulI* (sulfonamide resistance [26]), the *mphR(A)/mphA/mrx* gene cluster (macrolide/erythromycin resistance [27]), and *chrA* (chromate resistance [28, 29]). The *qacEΔ1* gene encodes a truncated and less-efficient cation efflux pump version of *qacE* (24).

Additionally, both segments included similarity to the *GNAT* (acetyltransferase) and *sul3* genes (30). The DNA sequence was 96% identical to *GNAT* from *Aeromonas hydrophila* with 72% query coverage (GenBank accession no. JX141473.1). The *sul3* gene was 100% identical with 55% query coverage (GenBank accession no. EF382672.1) yet included a 140-nucleotide 3′ deletion. The segments included the little-known *padR* transcriptional regulator. Nine additional ORFs were identified as hypothetical proteins.

A BLAST alignment of the contig derived from SRX560289 against all organisms resulted in 957 hits with an E value of $\leq 1 \times e^{-24}$ (31, 32). Among these, a single hit (GenBank accession no. CP014320.1) included the *dfrB4* mobile genetic element. It was a 130.6-kb plasmid from a clinical isolate of a patient with recurrent *E. coli* sequence type 131 (ST131) cystitis, the most prevalent *E. coli* lineage found in recurrent UTIs (33). Other hits included mismatches (≤15) and gaps (≤38) and excluded the *dfrB4* gene sequence. This suggests that the *dfrB4* mobile genetic element has recently been incorporated into the CP014320.1 multiresistance plasmid.

The DfrB4 protein confers TMP resistance. The high sequence identity of the DfrB4 protein to the well-characterized TMP-resistant DfrB1 protein (Table 1) and the 6-mm TMP zone of inhibition for these clinical samples (S. Salipante, personal communication) corresponding to an MIC of >16 μg/ml strongly argue that DfrB4 should confer TMP resistance to *E. coli* (34). To corroborate this hypothesis, the consensus *dfrB4* coding sequence was obtained (GeneArt gene synthesis; Thermo Fisher Scientific) with an *N*-terminal hexahistidine affinity tag for eventual purification. It was cloned into pET24-cTEM19m between NdeI and HindIII, under IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible overexpression in *E. coli* BL21(DE3). The MIC value was determined at least in duplicate through broth microdilution after incubation at 37°C for 14 to 16 h. We observed high resistance to TMP when expressing DfrB4 (>600 μg/ml, which is the maximal concentration of TMP that is soluble in 5% methanol) (35). The MICs for all negative controls were at least 2,000-fold lower, specifically *E. coli* BL21(DE3) (0.30 μg/ml) and *E. coli* BL21(DE3)/pET24-cTEM19m expressing an IPTG-inducible β-lactamase (0.075 μg/ml) (36). Thus, the MIC value of DrfB4 greatly surpasses the *E. coli* TMP resistance threshold (2 μg/ml) and is associated with 2.5% of all *E. coli* strains considered to exhibit dangerously high TMP resistance (≥512 μg/ml) (35, 37). In these experiments, DfrB4 was overexpressed; the expression level of DfrB4 in its native form is unknown.

To our knowledge, this work constitutes the first report of the type II TMP-resistant *dfrB4* gene identified in a clinical sample by whole-genome sequencing. The 2,000-fold increase in MIC for TMP that we observed in a transformed *E. coli* isolate points to TMP resistance in the clinical isolate identified carrying the *dfrB4* gene. The observation of this highly TMP-resistant DfrB4 within a known class I integron indicates that it is being disseminated, although the extent and rate of its propagation are currently unknown as it has not been systematically tracked. Our results highlight the importance of tracking the presence of this gene family in TMP-resistant clinical samples.

ACKNOWLEDGMENTS

We thank Steve Salipante for providing the zone of inhibition data for the original clinical samples and Simon Toulouse for assistance with drafting Fig. 1.

This work was supported by NSERC Discovery Grant 227853 (J.N.P). J.L.T. received a scholarship from Fonds Québécois pour la Recherche sur la Nature et le Technologies (FRQ-NT). This work was supported by funds from CIHR (A.R.M) (MOP 114879).

L.A. received a scholarship from PROTEO, the Québec Network for Research on Protein Structure, Function and Engineering, which is funded by FRQ-NT. J.L.T. and L.A. received scholarships from Faculté des études supérieures et post-doctorales de l'Université de Montréal (FESP).

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