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dfrA20, a Novel Trimethoprim Resistance Gene from *Pasteurella multocida*

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A novel trimethoprim resistance gene, designated *dfrA20*, was detected on the 11-kb plasmid pCCK154 from *Pasteurella multocida*. The *dfrA20* gene codes for a dihydrofolate reductase of 169 amino acids. Sequence comparisons revealed that the DfrA20 protein differed distinctly from all dihydrofolate reductases known so far.

Trimethoprim (TMP) competitively inhibits the enzyme dihydrofolate reductase, which is responsible for the reduction of dihydrofolate to tetrahydrofolate (6, 19). Bacterial resistance to trimethoprim can be intrinsic or acquired. Intrinsic resistance by either permeability barriers, folate auxotrophy, or dihydrofolate reductases with low affinity for TMP have been detected in various bacterial pathogens, including *Pseudomonas aeruginosa*, *Clostridium* spp., *Brucella* spp., *Bacteroides* spp., and *Enterococcus* spp. (15). Different types of acquired TMP resistance, including mutations in the promoter region or the dihydrofolate reductase structural gene, have been reviewed by Sköld (19). The most widespread TMP resistance mechanism, namely, the replacement of a TMP-sensitive dihydrofolate reductase by a plasmid-, transposon-, or cassette-borne TMP-resistant dihydrofolate reductase, causes high-level TMP resistance in various bacteria (6, 19). Up to now, more than 25 different TMP resistance-mediating dihydrofolate reductase (*dfr*) genes, subdivided on the basis of their structure into major types 1 and 2 (14), which nowadays are referred to as *dfrA* and *dfrB* (16), have been identified (17, 19). Although trimethoprim resistance is widespread among bacterial pathogens from human and animal sources, previous attempts to identify TMP resistance genes in bacteria of the genus *Pasteurella* have failed (3). It was assumed that bacteria of the genera *Pasteurella* and *Mannheimia* may carry *dfrA* or *dfrB* genes different from those previously identified in other gram-negative bacteria (8). In the present study, we describe a novel *dfr* gene, designated *dfrA20*, from *Pasteurella multocida*.

P. multocida strain GB154 was obtained from the nasal swab specimen of a calf suffering from pneumonia. Antimicrobial susceptibility testing (13) revealed that the strain was resistant to ampicillin (MIC, 32 μg/ml), sulfamethoxazole (MIC, 1,024 μg/ml), and trimethoprim (MIC, ≥128 μg/ml). Strain GB154 harbored a plasmid of 11 kb, designated pCCK154, which upon transformation into *Escherichia coli* JM109 (Stratagene, Amsterdam, The Netherlands) and electrotransformation into the plasmid-free and antibiotic-susceptible *P. multocida* field isolate B130 (9) proved to mediate resistance to sulfamethoxazole and trimethoprim. MICs, determined by broth macrodilution (13), were 1,024 μg of sulfamethoxazole/ml and ≥128 μg of trimethoprim/ml in both recipient strains. PCR assays for the most frequently detected *dfrA* and *dfrB* genes of gram-negative bacteria as described by Frech et al. (4) confirmed that none of these genes was present on plasmid pCCK154. However, a *sul2* gene, coding for a type II dihydropteroate synthase, was identified on this plasmid by PCR (4, 10). A restriction map of plasmid pCCK154 was constructed and served as a basis for cloning experiments. A ca. 1.9-kb PstI fragment was found to mediate trimethoprim resistance. This fragment plus another 0.7 kb upstream located on an EcoRV fragment of 5.6 kb and 1.2 kb downstream located on an EcoRV fragment of 5.4 kb were sequenced by primer walking (Fig. 1).

Sequence analysis of the ca. 3.8-kb segment revealed two stretches of 99% sequence identity between pCCK154 and the *Vibrio salmonicida* plasmid pRVS1 (GenBank accession number AJ289135). These homologous areas were from bases 293 to 1100 and 2179 to 3891 in the pCCK154 sequence (Fig. 1). The 1,078-bp sequence between these two pRVS1-homologous regions contained the novel TMP resistance gene. The *dfrA20* gene was bracketed by the 3′ end of a *thyA*-like gene whose deduced amino acid sequence showed 82% identity to the terminal 57 amino acids of a thymidylate synthase from *Cytophaga hutchinsonii* (accession number ZP00117986) and the 5′ end of a *topA*-like gene whose deduced amino acid sequence revealed 68% identity to the initial 119 amino acids of a type IA topoisomerase from *C. hutchinsonii* (accession number ZP00118579). The organization of a thymidylate synthase gene and a dihydrofolate reductase gene in the same operon has been observed in a number of bacteria, including, among others, *Bacillus subtilis* (7), *Bordetella bronchiseptica*, *Bordetella parapertussis*, and *Bordetella pertussis* (accession numbers NC 002927 to NC 002929). Both enzymes are essential for DNA synthesis in that thymidylate synthase catalyzes the transfer of a methyl group from N^5,N^{10}-methylentetrahydrofolate to deoxyuridylate, thereby generating deoxythymidylate and 7,8-dihydrofolate, whereas dihydrofolate reductase then converts 7,8-dihydrofolate to tetrahydrofolate, a precursor of N^5,N^{10}-methylentetrahydrofolate. At both junctions of pRVS1-homologous and -nonhomologous sequences in pCCK154, the 6-bp direct repeat ATACGT was detected (Fig. 1). The sequence ATACGT is part of the phosphoglucosamine mutase gene

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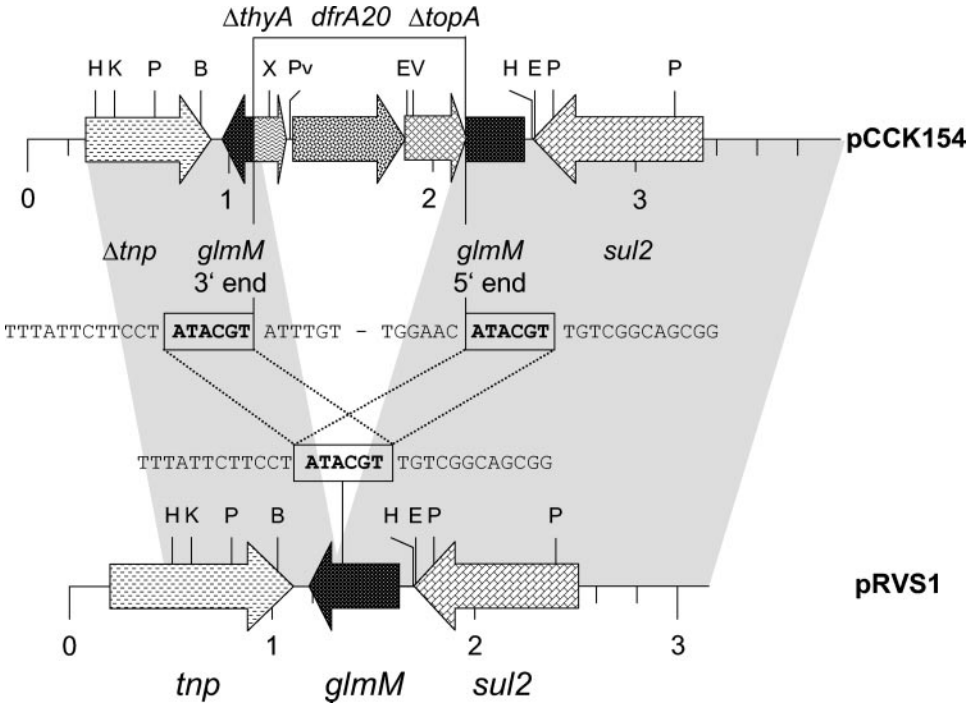

FIG. 1. Comparison of the *dfrA20-sul2* area of plasmid pCCK154 from *P. multocida* with the corresponding region of plasmid pRVS1. The arrows indicate the extents of the genes *tnp* (transposase), *glmM* (phosphoglucosamine mutase), *sul2* (sulfonamide-resistant dihydropteroate synthase), *dfrA20* (TMP-resistant dihydrofolate reductase), *thyA* (thymidylate synthase), and *topA* (type IA topoisomerase), with the arrowheads showing the directions of transcription. The prefix Δ means that the gene is truncated. The regions of homology between pCCK154 and pRVS1 are marked by grey shading. The sequences at the junctions of pRVS1-homologous and -nonhomologous parts in pCCK154 are shown in comparison to the corresponding pRVS1 sequence between the two maps. The 6-bp direct repeats are boxed. Restriction endonuclease cleavage sites are abbreviated as follows: B (BamHI), E (EcoRI), EV (EcoRV), H (HpaI), K (KpnI), P (PstI), Pv (PvuII), and X (XbaI).

glmM, which was disrupted in pCCK154 by the integration of the *dfrA20*-containing 1,078-bp segment (Fig. 1). Whether these 6-bp direct repeats represent relics of a transposable element that had been involved in the integration of the *dfrA20*-containing segment remains to be clarified. Bases 1 to 292 in the pCCK154 sequence did not reveal significant homology to sequences deposited in the databases.

The *sul2* gene was located in close proximity to the *glmM* gene in pCCK154 (Fig. 1). Its reading frame codes for a protein of 289 amino acids and thus represents the largest type II dihydropteroate synthase enzyme known to date. Previous studies showed that mutations in the terminal part of the *sul2* reading frame might cause an extension of the reading frame without having an impact on the functioning of the enzyme (1, 10). In the present case, the loss of a single A at position 793 within the *sul2* reading frame caused a frameshift mutation which led to the substitution of 6 codons and extended the reading frame by 18 codons compared to *sul2* from pRVS1. The high MIC of sulfamethoxazole indicates that these alterations in the C terminus had no negative impact on sulfonamide resistance.

The *dfrA20* gene is—to the best of our knowledge—the first TMP resistance gene detected in *P. multocida*. Analysis of the flanking regions did not reveal structures resembling gene cassettes (16). The deduced DfrA20 protein sequence consists of 169 amino acids and thus is in the same size range (152 to 189 amino acids) as most of the bacterial DfrA proteins known so far. Phylogenetic analysis showed that DfrA20 from *P. multocida* represents a separate branch in the phylogenetic tree and clusters with Dfr proteins from gram-positive bacteria such as *Staphylococcus*, *Bacillus*, and *Listeria* (Fig. 2). Identities to the known DfrA proteins determined on the basis of a multisequence alignment varied between 20.0 and 37.7%, with the highest levels of identity to the Dfr proteins being found for *Bacillus subtilis* (37.7%) (7) and *Staphylococcus haemolyticus* (37.0%) (2). This observation confirmed that DfrA20 is only distantly related to other DfrA proteins. In addition to the DfrA proteins shown in Fig. 2, there are numerous reading frames which have been identified during whole-genome sequencing and are assumed to code for dihydrofolate reductases. Their functional annotation, however, was usually based only on Conserved Domain Database and Clusters of Orthologous Groups assignments, not on experimental proof of their role in TMP resistance.

In contrast to the situation with tetracycline resistance genes (11) and macrolide-lincosamide-streptogramin B resistance genes (18), there is still no accepted nomenclature for the *dfrA* genes. Hall and Partridge addressed this problem and suggested that numbers should be assigned in the order of the database entries (5). Therefore, we tentatively designated the new *dfrA* gene from *P. multocida* as *dfrA20*. Since *dfrA20* is located on a small plasmid that also carries the sulfonamide resistance gene *sul2*, there is potential for this gene to be disseminated horizontally but also to be coselected by the use

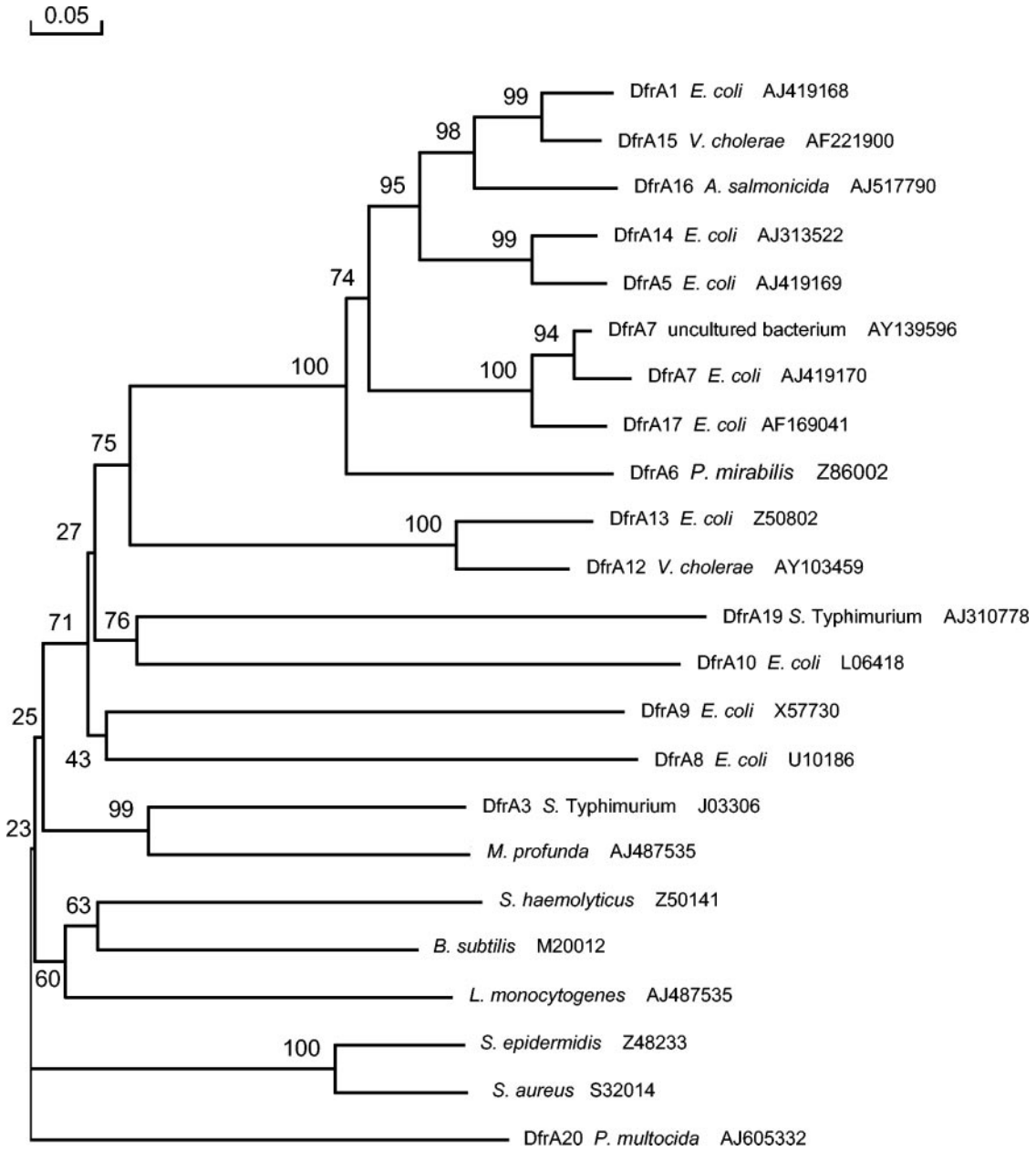

FIG. 2. Phylogenetic tree of the DfrA proteins involved in TMP resistance. Branch lengths are scaled according to amino acid exchanges observed in a multisequence alignment. The number at each major branch point refers to the percentage of times that a particular node was found in 10,000 bootstrap replications. The bacterial source and the GenBank accession number are given for each DfrA protein. For a number of DfrA proteins, e.g., DfrA1, several closely related sequences from different bacterial sources have been deposited in the databases. To reduce the complexity of this phylogenetic tree, only one representative for each type of DfrA protein was chosen. For this, the cutoff was set at ≥95% amino acid identity. According to the classification of the known Dfr proteins (also referred to as DHFR proteins in several database entries) into the two classes A and B (14, 16), all class A proteins for which numerals (arabic or roman) have been used in the database entries were indicated as DfrA protein followed by the respective arabic numeral. For the Dfr proteins from gram-positive bacteria which have not yet been assigned a number, only the bacterial source and the database accession number are given. DfrB proteins, which differ distinctly from DfrA proteins by their size and structure, have been excluded from this phylogenetic analysis. *V. cholerae*, *Vibrio cholerae*; *A. salmonicida*, *Aeromonas salmonicida*; *P. mirabilis*, *Proteus mirabilis*; *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; *M. profunda*, *Moritella profunda*; *L. monocytogenes*, *Listeria monocytogenes*; *S. epidermidis*, *Staphylococcus epidermidis*; *S. aureus*, *Staphylococcus aureus*.

of sulfonamides. The origin of the gene *dfrA20* remains to be answered. However, a lower GC content (35%) of the 1,078-bp segment containing *dfrA20* than of the whole genome of *P. multocida* strain Pm70 (41%) (12) suggested that *dfrA20* is most likely not an indigenous *P. multocida* gene.

Nucleotide sequence accession number. The sequence of a 3,891-bp segment of plasmid pCCK154 has been deposited in the EMBL database under accession number AJ605332.

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