

A New Sulfonamide Resistance Gene (*sul3*) in *Escherichia coli* Is Widespread in the Pig Population of Switzerland

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A new gene, *sul3*, which specifies a 263-amino-acid protein similar to a dihydropteroate synthase encoded by the 54-kb conjugative plasmid pVP440 from *Escherichia coli* was characterized. Expression of the cloned *sul3* gene conferred resistance to sulfamethoxazole on *E. coli*. Two copies of the insertion element IS15Δ/26 flanked the region containing *sul3*. The *sul3* gene was detected in one-third of the sulfonamide-resistant pathogenic *E. coli* isolates from pigs in Switzerland.

Sulfonamides have been widely used to treat bacterial and protozoal infections ever since their clinical introduction in 1935. To overcome the rapid emergence of resistance, sulfonamides have generally been combined since the 1970s with diaminopyrimidines (5, 6). The combination trimethoprim-sulfamethoxazole, for instance, is still commonly used in human medicine for the treatment of urinary tract infections. In veterinary medicine, sulfonamides alone or in combination with other antimicrobial compounds are widely used to prevent and treat diarrhea and other infectious diseases in intensive animal husbandry.

Sulfonamides compete with the structural analog *p*-amino-benzoic acid for binding to dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway, thus inhibiting the formation of dihydrofolic acid (17). Resistance to sulfonamides in *Escherichia coli* can result from mutations in the chromosomal DHPS gene (*folP*) (20, 23) or more frequently from the acquisition of an alternative DHPS gene (*sul*) (14, 19), whose product has a lower affinity for sulfonamides (21).

Only two alternative sulfonamide resistance DHPS genes (*sul1* and *sul2*) in gram-negative bacteria have been described to date (17). *sul1* and *sul2* from *E. coli* share 57% DNA identity, and their origin remains unknown, as their sequences are clearly distinct from all the known chromosomal DHPS genes from *E. coli* and other bacteria (14). Both genes seem to be distributed at equal frequency among sulfonamide-resistant *E. coli* isolates of clinical origin (15).

Pathogenic *E. coli* isolates from cases of neonatal and post-weaning diarrhea and edema diseases in pigs are frequently resistant to sulfonamides. In a recent study on pathogenic *E. coli* from animals in Switzerland, only 70% of the sulfonamide resistance in isolates from pigs could be explained by the presence of *sul1* or *sul2* genes (8). The mechanism of sulfonamide

resistance was unexplained for the remaining 30% of the isolates. The enterotoxigenic *E. coli* r10044 strain (LT, STb, and K88 positive), isolated from a case of diarrhea in a pig, was chosen to characterize the determinant responsible for sulfonamide resistance in these isolates. The name *sul3* has been inappropriately used for a DHPS gene found in both *Mycobacterium fortuitum* (9) and *Corynebacterium striatum* (GenBank accession no. AJ294721) whose protein is virtually identical to that of *sul1*, with the exception of four additional amino acids at its N terminus (6). Therefore, we chose to consider the new DHPS gene described below the third alternative DHPS gene conferring sulfonamide resistance in *E. coli* and named it *sul3*.

Analysis of the chromosomal DHPS (FolP) gene. To determine whether mutations in the *folP* gene were responsible for sulfonamide resistance, the *folP* gene of *E. coli* r10044 was amplified by PCR and sequenced as described previously (8). The DNA sequence was identical to that described by Lanz et al. (8) (GenBank accession no. AF483270). The amino acid sequence showed 100% identity to FolP of the sulfonamide-susceptible strain *E. coli* K-12 MG1655 (GenBank accession no. AE000398), confirming that the resistance was not due to a mutated *folP* gene.

Conjugal transfer of plasmid pVP440. Conjugations using *E. coli* r10044 as a donor and *E. coli* JF33 (a rifampin-resistant *E. coli* K-12 derivative [2]) as a recipient strain were performed as described previously (1). The transconjugants were selected on Mueller-Hinton agar plates containing 50 μg of rifampin and 200 μg of sulfamethoxazole per ml. A single 54-kb plasmid (pVP440) conferring sulfonamide resistance was transferred into the recipient strain at a frequency of 10⁻⁴ transconjugants per donor. Plasmid DNA was isolated from the transconjugants on Nucleobond AX cartridges by using the low-copy-number-plasmid extraction protocol recommended by the supplier (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The size of plasmid pVP440 was determined by restriction endonuclease analysis using *SphI* and *HindIII*. Small fragments were separated by classical agarose gel electrophoresis, and large fragments were separated by pulsed-field gel electrophoresis at 12°C in 1% SeaKem Gold agarose gel (BioWhittaker Molecular Applications Inc., Rockland, Maine) for 15 h at 200 V with pulse time ramping from 0.5 to 3.5 s in a

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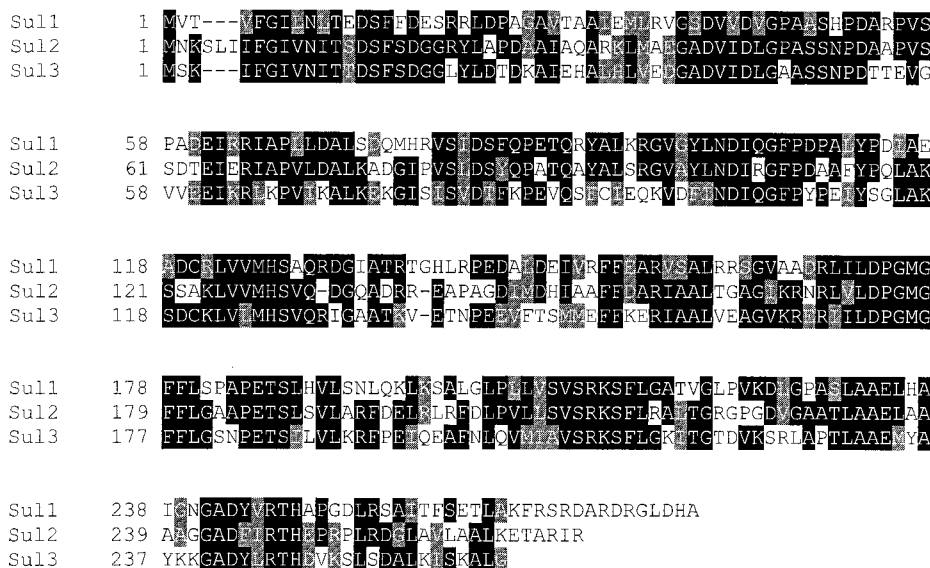


FIG. 1. Alignment of the amino acid sequence of the DHPSs Sul1, Sul2, and Sul3, conferring sulfonamide resistance. Sul1, DHPS of *E. coli* plasmid R388 (GenBank accession no. AF071413); Sul2, DHPS of *S. enterica* subsp. *enterica* plasmid pHCM1 (AL513383); Sul3, DHPS of *E. coli* plasmid pVP440 (AJ459418). The identical and similar amino acids are shaded black and gray, respectively.

CHEF-DR III electrophoresis unit (Bio-Rad Laboratories Inc., Hercules, Calif.). Plasmid pVP440 contained six *SphI* fragments with calculated sizes of 1.0, 2.0, 4.5, 6.0, 7.2, and 33 kb for a total size of 53.7 kb and seven *HindIII* fragments of 1.3, 1.6, 2.2, 9.1, 9.5, 14.1, and 15.8 kb for a total size of 53.6 kb. The size of pVP440 was estimated to be 54 kb.

Characterization of the *sul3* gene. Plasmid pVP440 was digested with the restriction enzyme *ClaI*, and the resulting fragments were randomly cloned into the vector pBluescript II SK(-) (Stratagene, La Jolla, Calif.). The ligated DNA was transformed into *E. coli* DH5 α (Life Technologies, Gaithersburg, Md.) by heat shock, and the transformants were selected on Mueller-Hinton agar containing 200 μ g of sulfamethoxazole per ml. The sulfonamide-resistant transformants harbored plasmid pBVP44, which was generated by the insertion of a 3-kb *ClaI* fragment from pVP440 into pBluescript II SK(-). The 3-kb DNA insert was sequenced and found to contain a 789-bp gene (*sul3*) that specifies a putative 263-amino-acid protein (Sul3) with a calculated molecular mass of 28.9 kDa. Putative -35 and -10 promoter sequences exist within the 136 bp preceding the ATG start codon at positions 2841 and 2862, respectively. Similarity searches of protein data banks using BLAST (National Center for Biotechnology Information) and LALIGN (4) revealed evident homologies of Sul3 with DHPSs. There were amino acid identities of 50.4% overall to Sul2 from *Salmonella enterica* subsp. *enterica* plasmid pHCM1 (12), 40.6% to Sul2 from *E. coli* plasmid RSF1010 (16), and 40.9% to Sul1 from *E. coli* plasmid R388 (19). Based on amino acid homology (Fig. 1) and phenotype, Sul3 was considered a new sulfonamide-resistant DHPS.

Phenotypic expression of *sul3*. The region containing only the *sul3* gene and its putative promoter sequence was amplified by PCR from plasmid pVP440 by using *Taq* DNA polymerase (Roche Diagnostics AG, Basel, Switzerland) and an annealing temperature of 51°C. *XbaI* and *PstI* restriction sites were in-

corporated into forward (*sul3*PF, 5'-CATTCTAGAAAACAGTCGTAGTTCG [positions 2780 to 2797 in AJ459418]) and reverse (*sul3*R, 5'-CATCTGCAGCTAACCTAGGGCTTTGGA [positions 3770 to 3753]) primers to facilitate directional cloning into pUC19. In the resulting plasmid, pUVP4401, *sul3* is placed in the direction opposite that of the *lacZ* promoter of pUC19 and is under the control of its own promoter. MICs were determined by broth microdilution tests according to NCCLS guidelines (11). The expression of *sul3* on plasmid pUVP4401 conferred increased resistance to sulfamethoxazole in *E. coli* JF33, with the MIC being higher than 1,024 μ g/ml, whereas JF33 alone or harboring the vector pUC19 remained susceptible to sulfamethoxazole, with the MIC being 8 μ g/ml. MICs higher than 1,024 μ g of sulfamethoxazole per ml were also observed for the wild-type strain *E. coli* r10044 and the JF33 transconjugant harboring the sulfonamide resistance plasmid pVP440. No chloramphenicol resistance was observed (MIC \leq 2 μ g/ml) for *E. coli* JF33 and JF33 harboring plasmid pVP440, confirming the nonfunctionality of the truncated *cmfA1* gene (3) on plasmid pVP440 (see below).

Sequence and structure of the *sul3*-flanking regions. The regions flanking the left and the right sides of the *sul3* gene were sequenced from two large *SphI* fragments from pVP440 cloned into pUC19 (pUVP444 and pUVP445) and overlapping with the *ClaI* insert of pBVP44 (Fig. 2). DNA analysis of the *sul3*-flanking regions revealed distinct domains with strong homologies to DNA sequences of diverse origins. A 3.6-kb domain contained the *sul3* gene, a putative oxidoreductase gene (*orf1*), and the partial sequence of a new putative insertion element (IS440). This domain was embedded between two copies of the insertion element IS15 Δ /26. These IS15 Δ /26 copies separate the *sul3* domain from DNA sequences almost identical (one single base change) to plasmid pHCM1 of the multidrug-resistant *S. enterica* subsp. *enterica* serovar Typhi strain CT18 (GenBank accession no. AL513383) (12) on the

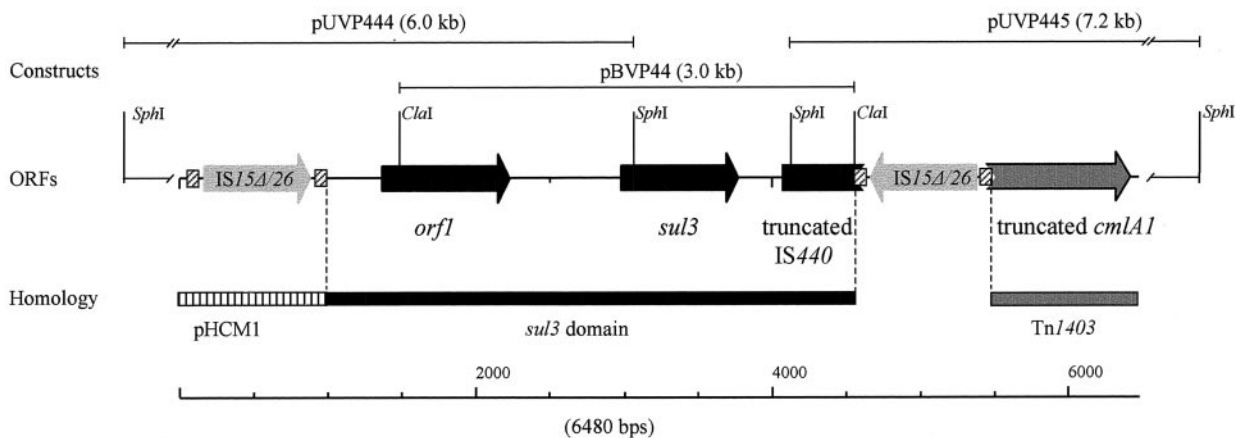


FIG. 2. Organization of the region from plasmid pVP440 containing the sulfonamide resistance gene *sul3*. The *sul3* domain and the pHCM1-like and Tn1403-like sequences are labeled; the different domains are separated with vertical dotted lines. The open reading frames (ORFs) of the *sul3* domain are represented by black arrows (*orf1*, putative oxidoreductase gene; *sul3*, sulfonamide-resistant DHPS gene, IS440, truncated putative insertion element) and the ORF of the truncated *cmlA1* gene (chloramphenicol efflux gene) is represented by a gray arrow. The two copies of the insertion sequence IS15 Δ /26 are represented by gray arrows, with inverted repeats indicated by hatched squares. The fragments of pVP440 that were cloned into pUC19 (pUVP444 and pUVP445) and into pBluescript II SK(-) (pBVP44) and used for sequence analysis are depicted by narrow lines.

left side and from DNA sequences almost identical (one single base change) to Tn1403 from *Pseudomonas aeruginosa* (GenBank accession no. AF313472) (13) on the right side (Fig. 2). Mobile genetic elements containing antibiotic resistance genes flanked by two copies of IS15 Δ /26 have already been described (10, 18). Two direct repeats of IS15 Δ /26 flanking the kanamycin resistance transposon Tn2680 have been shown to mediate cointegration in the *E. coli* chromosome, generating an 8-bp target site duplication (7). This suggests that the *sul3* gene could be borne on a composite element flanked by IS15 Δ /26. However, the 8-bp direct repeats were not present outside the flanking IS15 Δ /26.

The putative oxidoreductase (*orf1*) of the *sul3* domain showed the highest amino acid identity (45.5%) with the oxygenase-like protein Aur2G from *Streptomyces aureofaciens* (GenBank accession no. AY033994). The putative IS440, whose incomplete transposase shared 53% amino acid identity with that of IS406 from *Burkholderia cepacia* (GenBank accession no. P24575) (24), appeared to be interrupted by the insertion of an IS15 Δ /26 element. From there, the DNA was 100% identical to the DNA of IS15 Δ /26 from transposon Tn1525 (GenBank accession no. M12900) (22). A partial chloramphenicol resistance efflux protein CmlA1 (3) from Tn1403 lacking the first 86 amino acids was identified downstream of IS15 Δ /26. Similar to the 3' end of the putative IS440, the 5' end of *cmlA1* gene has apparently been truncated by the insertion of IS15 Δ /26.

Detection and distribution of *sul3* in the pig flora. Twenty-seven sulfonamide-resistant *E. coli* isolates, from pigs, that did not hybridize with specific DNA probe for *sul1* and *sul2* (8) were examined for the presence of *sul3*. Twenty-five of them were enterotoxigenic strains from cases of neonatal and post-weaning diarrhea, and two were verotoxigenic strains of serogroups O139 and O141 (r10066 and r10281) from cases of edema disease (8). The *sul3* gene was detected in all 27 strains by PCR using two oligonucleotide primers specific to *sul3* (*sul3F*, 5'-GAGCAAGATTTTGGGAATCG [positions 2981

to 3000 in AJ459418] and *sul3R*, 5'-CATCTGCAGCTAACC TAGGGCTTTGGA [positions 3770 to 3753]) and an annealing temperature of 51°C. The presence of *sul3* in these 27 isolates was confirmed by dot blot DNA hybridization as described previously (8) using a PCR probe for *sul3* labeled by random priming using the Dig-High Prime kit (Roche Diagnostics). *E. coli* strain r10637 (laboratory collection), which harbors both *sul1* and *sul2*, was used as a negative control for both PCR and dot blot hybridization analyses (data not shown).

Restriction analysis with *SphI* of the *sul3*-carrying conjugative plasmid of a second epidemiologically unrelated enterotoxigenic *E. coli* strain (r10096) showed a profile identical to that of pVP440. This suggests either the presence of a pVP440-carrying enterotoxigenic *E. coli* clone in the pig population or the horizontal spread of pVP440 between *E. coli* strains. Additional conjugations were attempted with the verotoxigenic *E. coli* strains r10066 and r100281. *E. coli* r10281 transferred a *sul3*-harboring plasmid (pVP2810) larger than pVP440 and different in its *SphI* restriction profile into the recipient strain JF33. No transconjugants were obtained with *E. coli* r10066. However, DNA-DNA hybridization analyses showed that the *sul3* gene from *E. coli* r10066 was also located on a plasmid with a size similar to that of pVP2810 (data not shown).

In conclusion, our study describes a new sulfonamide resistance determinant called *sul3*, probably acquired by *E. coli* from a distantly related organism. The mobility of the *sul3* element was not strictly demonstrated in the present study. However, the structure of the *sul3* domain with its flanking IS15 Δ /26 copies and the presence of *sul3* on different plasmids in different *E. coli* clonal lineages suggest that this resistance determinant has a strong potential for a large diffusion within the bacterial population.

The discovery of the new antimicrobial resistance determinant *sul3* demonstrates once more the wide ability of bacteria to adapt to hostile environments. The appearance of new resistance genes, even toward old antimicrobial agents like sul-

fonamides, on conjugative plasmids and transposable elements should stress the importance of the appropriate and more prudent use of antibiotics in both public health and agriculture.

Nucleotide sequence accession number. The *sul3* gene and the flanking regions have been assigned EMBL accession number AJ459418.

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