Characterization of sulphonamide-resistant *Escherichia coli* using comparison of *sul2* gene sequences and multilocus sequence typing

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The *sul2* gene encodes sulphonamide resistance (Sul^R) and is commonly found in *Escherichia coli* from different hosts. We typed *E. coli* isolates by multilocus sequence typing (MLST) and compared the results to sequence variation of *sul2*, in order to investigate the relation to host origin of pathogenic and commensal *E. coli* strains and to investigate whether transfer of *sul2* into different genomic lineages has happened multiple times. Sixty-eight *E. coli* isolated in Denmark and Norway from different hosts and years were MLST typed and *sul2* PCR products were sequenced and compared. PFGE was performed in a subset of isolates. All isolates were divided into 45 different sequence types (STs), with clonal complexes CC10, CC23, CC168, CC350 and CC69 being the most frequent. The *sul2* gene from the majority of *E. coli* strains had only two point mutations, at positions 159 and 197, leading to a synonymous and a non-synonymous change, respectively. Five strains had extra single mutations. All poultry, poultry meat, and Danish human blood isolates had the same *sul2* ST and some of these strains clustered under the same MLST STs, indicating that they shared habitats. Most PFGE profiles clustered according to source, but some included different sources. Sul^R *E. coli* from different animals, food, human faeces and infections did not cluster according to their origin, suggesting that these habitats share *E. coli* and *sul2* gene types. However, while pig isolates on one occasion clustered with urinary tract infection isolates, poultry isolates seemed more related to isolates from bloodstream infections in humans. Presence of mainly two types of the *sul2* gene in both human and animal isolates, irrespective of date and geography, and the presence of both types in the same clonal lineages, suggest horizontal transfer of *sul2*.

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Abbreviations: CC, clonal complex; DHPS, dihydropteroate synthase; MLST, multilocus sequence typing; ST, sequence type; UPGMA, unweighted pair group method using averages; UTI, urinary tract infection.

A supplementary figure and two supplementary tables are available with the online version of this paper.

INTRODUCTION

The known genes encoding plasmid-borne sulphonamide resistance (Sul^R) are *sul1*, *sul2* and *sul3*; each encodes a drug-resistant dihydropteroate synthase (DHPS) enzyme. Among them, the *sul2* gene has previously been reported as

the most prevalent one in *Escherichia coli* from pig and pork, poultry and poultry meat, cow and beef, mutton, human faeces and urinary tract infections (UTIs) (Blahna *et al.*, 2006; Gow *et al.*, 2008; Grape *et al.*, 2003; Hammerum *et al.*, 2006; Kerrn *et al.*, 2002; Sunde & Norstrom, 2006; Trobos *et al.*, 2008). Based on a few partial sequences, this gene seems highly conserved, whether it is carried on small non-conjugative or large conjugative resistance plasmids (Radstrom & Swedberg, 1988; Sorum & L'Abée-Lund, 2002). It has been suggested that horizontal gene transfer plays a much larger role than clonal expansion in the spread of trimethoprim-sulfamethoxazole resistance (Blahna *et al.*, 2006), but there is a need to investigate this further.

Multilocus sequence typing (MLST) is used to characterize *E. coli* isolates, among other bacteria, on the basis of sequence variation of seven housekeeping loci. The different nucleotide sequences of each locus are assigned different allele numbers, and then each strain is defined by the alleles of the seven loci (allelic profile). Closely related organisms can be grouped in clonal complexes. Analyses of *E. coli* isolates by MLST help to determine the genetic relatedness of the isolates, which is important for molecular epidemiological and evolutionary studies (Feil *et al.*, 2004). MLST is used to study the population biology of micro-organisms and provides an understanding of the population structure of important pathogens such as *E. coli*. Isolates that exhibit similar or identical MLST genotypes are very closely related and have descended from a recent common ancestor (Turner & Feil, 2007).

In the present study, we applied MLST, followed by PFGE typing, to characterize *sul2*-positive strains of *E. coli* from different sources, and sequenced the *sul2* gene, with the following aims: (1) to see if the same clonal complexes contained resistant strains from different sources, which would be an indication that resistant strains are transferred between niches, and (2) to investigate whether horizontal gene transfer of the *sul2* gene seems to have happened frequently.

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METHODS

Bacterial isolates. A total of 68 *E. coli* from different sources (pig, pork, poultry, poultry meat, mutton, healthy humans, blood stream infections and UTI), years (1969–2004) and countries (Denmark and Norway) were chosen for the study (Table 1). Except for the human blood isolates and the Norwegian animal isolates, the isolates were obtained from other studies (Hammerum *et al.*, 2006; Kerrn *et al.*, 2002; Sunde & Norstrom, 2006; Trobos *et al.*, 2008). They were selected by their resistance to sulphonamides and the presence of the *sul2* gene. A breakpoint of $\geq 512 \mu\text{g ml}^{-1}$ was used for determining sulphamethoxazole resistance according to the Clinical Laboratory Standards Institute (CLSI).

MLST. We used the MLST protocol standardized for *E. coli* maintained at the Max Planck Institute for Infection Biology website (<http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli/documents/>

Table 1. Variations in the *sul2* gene of *E. coli* strains of animal, meat and human origin

Source	Year	Country	No. of strains	No. of strains*		
				<i>sul2</i> (a)	<i>sul2</i> (b)	<i>sul2</i> (c)
Human						
Blood infections	1969	Denmark	6	6	0	0
	2002	Norway	5	2	3	0
UTI	2000	Denmark	5	2	3	0
Healthy (faeces)	1997, 2002	Denmark	13	6	5	2
Pig						
Faeces	2002	Norway	5	3	2	0
	2002, 2003	Denmark	6	3†	3	0
Meat	2002	Norway	5	5‡	0	0
	2002, 2003	Denmark	4	2	2	0
Poultry						
Faeces	2002	Norway	3	3	0	0
	2004	Denmark	5	5	0	0
Meat	2002	Norway	5	5	0	0
	2004	Denmark	4	4§	0	0
Mutton	2001	Norway	2	1	1	0

*Number of strains belonging to one of the three *sul2* gene variants [*sul2*(a), *sul2*(b) and *sul2*(c)]. Mutations are at positions 159, 197 and 427.

†One isolate contains an extra point mutation at position 672 (T instead of G).

‡One isolate contains an extra point mutation at position 288 (A instead of G).

§One isolate contains an extra point mutation at position 489 (A instead of T).

primersColi_html). When two possible primers were given for the same direction, we chose the following: *fumCR*, *gyrBR*, *purAF*, *recAF* and *recAR*.

Sequencing of the PCR products was performed using the services of MacroGen. Sequences of the seven housekeeping genes (*adh*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*) were analysed by BioNumerics using a special plug-in for MLST of *E. coli*.

PCR and sequencing of the *sul2* gene. Presence of the *sul2* gene was investigated by PCR using primers *Sul2*-F (5'-TTT CGG CAT CGT CAA CAT AA-3') and *Sul2*-R (5'-GTG TGT GCG GAT GAA GTC AG-3') (primer concentration 10 µM). A multiplex PCR kit (Qiagen) was used with the following amplification conditions: heating for 15 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 48 °C for 90 s and 72 °C for 90 s, followed by 72 °C for 10 min. *E. coli* NCTC 50020 was used as positive control and *E. coli* DH5α plasmid pUVP4401 was the negative control.

The *sul2* PCR products (727 bp) were purified by using the NucleoFast 96 PCR plates (Macherey-Nagel) and sequenced at MacroGen. The sequences were analysed with KODON software (Applied-Maths) and compared by multiple alignment using CLUSTAL W and MEGA4 software (Tamura *et al.*, 2007; Thompson *et al.*, 1997).

PFGE. PFGE was used to type *E. coli* from poultry, poultry meat and Danish blood infections. The one-day PFGE Standardized Laboratory Protocol for Molecular Subtyping of *E. coli* O157:H7 from PulseNet (http://www.cdc.gov/pulsenet/protocols/ecoli_salmonella_shigella_protocols.pdf) was used with the following variation: the restriction enzyme used was *SpeI* (20 U µl⁻¹) (5 µl 10 × buffer 2, 42 µl sterile water, 1 µl BSA and 2 µl *SpeI*). Digestions were performed in 50 µl volumes.

Computer-assisted analysis of *SpeI* profiles was performed using the BioNumerics software (Applied Maths). The dendrogram was constructed using Dice similarity coefficients of *SpeI* PFGE patterns and the UPGMA clustering method (see Supplementary Fig. S1, available with the online version of this paper).

RESULTS AND DISCUSSION

Diversity of *sul2*-positive *E. coli* STs among animals, food, humans and infections

A minimal spanning tree (MS_{TREE}) shows the distribution of the multilocus sequence types (STs) and their relation to the *sul2* gene variants (see below) of the 68 *E. coli* isolates from all the sources (see Fig. 1).

Among the 68 *E. coli* isolates studied, 45 different STs were identified (Supplementary Table S1). The largest group of isolates (24%) was part of the clonal complex ST10 (CC10). They originated from pigs (*n*=2), pork (*n*=2), poultry (*n*=3), poultry meat (*n*=2), healthy humans (*n*=6) and blood infection (*n*=1). This was not surprising, since CC10 is the biggest clonal complex of the species, and *E. coli* isolates from very different hosts are assigned to it (e.g. healthy human, UTI, septicaemia, diarrhoea, pig, dog, chicken and soil) (<http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli/GetTableInfo.html>). CC10 comprised mainly the non-pathogenic *E. coli* isolates of our study. One-third of the pig (3 out of 9) and pork (4 out of 11) isolates

belonged to the clonal complex CC23. A UTI isolate was also associated with this group.

Seven isolates were part of CC168; they were isolated from poultry (*n*=1), pig (*n*=2), pork (*n*=1), blood infections (*n*=2) and UTI (*n*=1). CC350 contained *E. coli* from poultry meat (*n*=2) and blood infection (*n*=1). Three isolates from poultry meat, a healthy human and a blood infection belonged to CC69. This clonal complex is mainly represented by human UTI and bacteraemia *E. coli* isolates, although it has also been found in cow and sheep isolates (Tartof *et al.*, 2005).

Fig. 2 shows an eBURST diagram of the current *E. coli* MLST global population structure. The spatial distribution of Sul^R *E. coli* in the population seemed random; however, we have not tested this statistically. The Sul^R strains typed in this study were scattered among the rest of the Sul^R and Sul^S *E. coli* from all over the world. This indicates that there is not a cluster of this specific resistant population, and that all *E. coli* could potentially become Sul^R.

Comparison of *sul2* gene sequences

In total six point mutations were recognized (Fig. 3). We decided to name these variants of the *sul2* genes in the following manner: if a mutation resulted in an amino acid change in the DHPS protein we gave it a new letter. Extra non-synonymous mutations are simply named as the *sul2* variant they derive from.

The first mutation, at position 159, was a silent thymine (T) or cytosine (C) third-codon substitution. The second, at position 197, was a C or adenine (A) second-codon non-silent substitution leading to alanine or glutamic acid, respectively. Almost all of the *E. coli* strains tested fell into one of these two variants: *sul2*(a) (TC variant) or *sul2*(b) (CA variant) (GenBank accession numbers FJ200242 and FJ200243, respectively) (Table 1). Two isolates, within the TC variant, contained an extra non-synonymous mutation at position 427 (A instead of G); this mutation was named *sul2*(c) (GenBank accession number EU653286).

The *sul2*(a) variant was the most frequently found among the strains tested. Three strains with the *sul2*(a) variant contained an extra synonymous mutation at position 288, 489 or 672 (GenBank accession numbers EU653287, EU653288 and EU653289, respectively) (see Fig. 3).

This study confirms, as previously reported on a smaller sample (Radstrom & Swedberg, 1988) that the *sul2* gene is highly conserved, with few point mutations, though not identical as was previously concluded (Sorum & L'Abée-Lund, 2002). Presence of only two main types of the *sul2* gene in both human and animal isolates, irrespective of time and geography, suggests horizontal transfer of the gene. It has been concluded by others that this had probably happened within a short time, since signs of more point mutations, indicative of genetic drift, were not found (Sorum & L'Abée-Lund, 2002). However, there may also be

Fig. 1. Distribution of MLST STs and their relation to the *sul2* gene variants within a minimal spanning tree (MS_{TREE}) of 68 *Escherichia coli* from various sources. Each ST is represented by a circle. Ellipses containing several STs indicate clonal complexes (CC). The lines connecting the STs within a clonal complex are shaded in colours. Black lines connecting pairs of STs indicate that they differ in one allele (thick lines), two alleles (thin), or three to seven alleles (dotted). Green circles represent isolates that contain the *sul2*(a) gene variant, red circles represent the *sul2*(b) variant and blue circles represent both variants [except ST10, which contains the three variants: *sul2*(a), *sul2*(b) and *sul2*(c)]. S, swine; PK, pork; P, poultry; PM, poultry meat; HH, healthy human; BI, blood infection; UTI, urinary tract infection.

a second possible explanation, namely in order to function efficiently as a DHPS enzyme, the protein cannot tolerate many changes to its amino acid sequence. Therefore, it could also have been that an ancient transfer occurred which resulted in a wide dissemination.

The two main *sul2* STs were present in the same clonal lineage. This indicates that horizontal gene transfer has happened several times, into the same clonal lineage, i.e. spread of *sul2* happens independently of the evolution of the clonal lineage.

Link between poultry, poultry meat and human blood *E. coli* isolates and their *sul2* gene ST

Interestingly, all poultry, poultry meat, and Danish human blood isolates had the same *sul2*(a) variant and some of these strains clustered under the same multilocus STs (ST10, ST57 and ST69) (Supplementary Tables S1 and S2).

These isolates were PFGE typed in order to dismiss clonality (data not shown). We found that many PFGE profiles clustered ($\geq 80\%$ genomic similarity) (Supplementary Fig. S1). Most clustered according to their source, but there were exceptions to this (Supplementary Fig. S1). One cluster associated two poultry isolates, two isolates from poultry meat and one from a blood infection. Another linked one poultry strain with five human blood isolates. This is an indication that poultry isolates of *E. coli* may be a source of extra-intestinal *E. coli* infection in humans; however, investigations with more strains are needed to confirm this.

Transfer mechanism of *sul2*

Resistance genes can spread via two phenomena, horizontal gene transfer and clonal expansion. In this study, we found

Fig. 2. Population snapshot of *E. coli* clusters of related and unrelated STs within the entire *E. coli* MLST database (performed using eBURST v3). This diagram compares the STs identified in this study (*Sul^R E. coli*, with pink haloes) with all the *E. coli* isolates in the MLST database (*Sul^S* and *Sul^R*). It does not show the genetic distance between unrelated STs and CCs. In each CC the primary founder is at a central position. A node between two STs represents a single-locus variation. [Performed on 11 September 2008.]

the same MLST STs, clonal complexes and *sul2* sequence among *E. coli* isolates from very different ecological niches, regions and years, just as we found the same *sul2* gene variant among different MLST types. In the first case, this suggests that strains were of clonal origin with a stable

presence of *sul2*, while the other case indicates a widespread horizontal transfer of *sul2* between different hosts. It may be that clonal lineages differ in their ability to act as recipients for horizontally transferred genes, in particular plasmid-encoded genes such as *sul2*.

	159		197	427	
sul2(a)	...	aa t ccc gac gcc gcg cct gtt tcg tcc gac aca gaa atc gcg ...	gct ...	FJ200242	
sul2(b)	...	aa c ccc gac gcc gcg cct gtt tcg tcc gac aca gaa atc gag ...	gct ...	FJ200243	
sul2(c)	...	aa t ccc gac gcc gcg cct gtt tcg tcc gac aca gaa atc gcg ...	act ...	EU653286	
Extra synonymous mutations of <i>sul2(a)</i> with positions and GenBank acc. no.:					
<i>sul2(a)</i> variant	288	...	tt a ...	EU653289	
<i>sul2(a)</i> variant	489	...	gg a ...	EU653287	
<i>sul2(a)</i> variant	672	...	cc t ...	EU653288	

Fig. 3. Mutation sites in the sequence of the *sul2* gene: representation of the three *sul2* gene variants [*sul2(a)*, *sul2(b)* and *sul2(c)*]. Mutations are at positions 159, 197 and 427. The extra mutations in three isolates derive from the *sul2(a)* type. These isolates contain an extra point mutation at position 288 (A instead of G), position 489 (A instead of T) and position 672 (T instead of G), respectively.

To conclude, this study shows that the same type of *E. coli* carrying an identical *sul2* gene can colonize both animals and humans, and that strains which can be found among animals may be implicated in human infections such as UTI and septicæmia. Whether the strains or the *sul2* gene transfer from animals to humans, or vice versa, cannot be assessed from the current study, due to the lack of a specific *sul2* type colonizing each of the hosts.

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