J Antimicrob Chemother 2017; **72**: 3317–3324
doi:10.1093/jac/dkx327 Advance Access publication 18 September 2017

Journal of Antimicrobial Chemotherapy

Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in *d*-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B

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Received 23 June 2017; returned 17 July 2017; revised 4 August 2017; accepted 4 August 2017

Objectives: Plasmid-mediated mobilized colistin resistance is currently known to be caused by phosphoethanolamine transferases termed MCR-1, MCR-2, MCR-3 and MCR-4. However, this study focuses on the dissection of a novel resistance mechanism in *mcr-1*-, *mcr-2*- and *mcr-3*-negative *d*-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (*Salmonella* Paratyphi B *d*Ta+) isolates with colistin MIC values >2 mg/L.

Methods: A selected isolate from the strain collection of the German National Reference Laboratory for Salmonella was investigated by WGS and bioinformatical analysis to identify novel phosphoethanolamine transferase genes involved in colistin resistance. Subsequently PCR screening, S1-PFGE and DNA-DNA hybridization were performed to analyse the prevalence and location of the identified *mcr-5* gene. Cloning and transformation experiments in *Escherichia coli* DH5α and *Salmonella* Paratyphi B *d*Ta+ control strains were carried out and the activity of MCR-5 was determined *in vitro* by MIC testing.

Results: In this study, we identified a novel phosphoethanolamine transferase in 14 *mcr-1*-, *mcr-2*- and *mcr-3*-negative *Salmonella* Paratyphi B *d*Ta+ isolates with colistin MIC values >2 mg/L that were received during 2011–13. The respective gene, further termed as *mcr-5* (1644 bp), is part of a 7337 bp transposon of the Tn*3* family and usually located on related multi-copy ColE-type plasmids. Interestingly, in one isolate an additional subclone with a chromosomal location of the *mcr-5* transposon was observed.

Conclusions: Our findings suggest that the transfer of colistin-resistance-mediating phosphoethanolamine transferase genes from bacterial chromosomes to mobile genetic elements has occurred in multiple independent events raising concern regarding their variety, prevalence and impact on public health.

Introduction

Colistin (polymyxin E) is a cationic antimicrobial peptide that interacts with the lipid A portion of LPS molecules in the outer membrane of Gram-negative bacteria leading to disruption of bacterial membranes and cell death.[1,2] Colistin is considered as one of the last-resort antibiotics against human infections caused by MDR Gram-negative bacteria including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and carbapenemase-producing Enterobacteriaceae. However, colistin is also commercialized in veterinary medicine and broadly used to control intestinal infections in food-producing animals such as pigs and poultry.[1–3] The excessive and prolonged use of colistin in both animals and humans corresponds with increased colistin MIC (non-WT phenotype) rates in Enterobacteriaceae.[4] Resistance against colistin is commonly caused by modification of lipid A molecules in the outer bacterial membrane LPS portion through addition of phosphoethanolamine or 4-amino-4-deoxy-L-arabinose residues. These modifications lead to a lower net negative charge of the LPS resulting in reduced binding of colistin and therefore in decreased bacterial susceptibility.[2,5–7]

Until recently most bacterial resistance mechanisms against colistin were assumed to be linked to overexpression of LPS-modifying genes due to chromosomal point mutations and therefore not transferable via horizontal gene transfer.[6–9] However, in 2015 Liu *et al.*[9] described the first plasmid-located colistin resistance gene (*mcr-1*) belonging to the phosphoethanolamine transferase enzyme family. This gene was detected in *Escherichia coli* isolates recovered from animals and patients in China.[9] Following this finding a number of publications reported the occurrence of *mcr-1* in Enterobacteriaceae isolated from food, animals and humans.[10] Recent announcements describe the emergence of

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mcr-1 variants through point mutations and in sequence databases (i.e. GenBank) different variants of the *mcr-1* gene identified in *E. coli*, *Klebsiella* and *Salmonella* diverging in eight distinct alleles are available.[11–13] In 2016, another distantly related mobilizable colistin resistance gene termed *mcr-2* was discovered in porcine and bovine *E. coli* isolates from Belgium[14] and a short time ago the *mcr-3* and *mcr-4* genes were detected and added to the list of mobile phosphoethanolamine transferase genes causing colistin resistance in Enterobacteriaceae.[15,16]

Among the reported *mcr-1*-positive Enterobacteriaceae isolates of *d*-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (*Salmonella* Paratyphi B *d*Ta+), formally called *Salmonella* Paratyphi B variant Java, are described.[17,18] In Germany, Belgium and the Netherlands an MDR lineage of *Salmonella* Paratyphi B *d*Ta+ belonging to ST 28 persists in the poultry production since the 1990s.[19] Between 2011 and 2016, 86 (21%) of 414 tested *Salmonella* Paratyphi B isolates from the strain collection of the German National Reference Laboratory for the Analysis and Testing of Zoonoses (NRL Salmonella) were determined to have a non-WT phenotype for colistin (MIC >2 mg/L). PCR screening revealed that 54 isolates carried the *mcr-1* and none of them the *mcr-2* or the *mcr-3* gene. Of the remaining 32 strains, 12 isolates showed a non-WT phenotype for ampicillin, ciprofloxacin, colistin, nalidixic acid, sulfamethoxazole, tetracycline, trimethoprim and in some cases tigecycline. This phenotypic pattern was unique to strains with unknown colistin resistance mechanism. The aim of this study was to identify genetic colistin resistance determinants in these strains.

Materials and methods

Selection of Salmonella Paratyphi B dTa+ strains

This study was primarily focusing on 12 *mcr-1*-, *mcr-2*- and *mcr-3*-negative *Salmonella* Paratyphi B *d*Ta+ isolates from the German NRL Salmonella showing a non-WT phenotype for ampicillin, ciprofloxacin, colistin, nalidixic acid, sulfamethoxazole, tetracycline, trimethoprim and occasionally tigecycline. The isolates were received for diagnostic serotyping and initially isolated from poultry and food thereof by public or private diagnostic laboratories across Germany in the years 2011–13. The study was later extended to 20 additional isolates with unknown colistin resistance mechanisms received in the years 2011–16.

WGS

Bacteria were cultivated on LB agar. A single colony was inoculated in liquid LB supplemented with a final concentration of 2 mg/L colistin sulphate (Sigma, Darmstadt, Germany) or 100 mg/L ampicillin (Sigma) if required and cultivated under shaking conditions (180–220 rpm) at 37 °C for 14–16 h. DNA from bacterial cells was isolated from liquid cultures using the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Sequencing libraries were prepared with the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Paired-end sequencing was performed in 2×251 cycles on the Illumina MiSeq benchtop using the MiSeq Reagent v3 600-cycle Kit (Illumina).

Identification of a novel genetic colistin resistance determinant based on WGS data

To identify novel resistance determinants, bioinformatic analysis of sequence data obtained for one randomly selected *Salmonella* Paratyphi B *d*Ta+ isolate (13-SA01718) with a non-WT phenotype for ampicillin, ciprofloxacin, colistin, nalidixic acid, sulfamethoxazole, tetracycline and trimethoprim was carried out using CLC Genomics Workbench 9.5.2 (Qiagen, Hilden, Germany) and *RASTtk*[20] annotation. Raw sequencing data were trimmed and subjected to read mapping against the complete bacterial chromosome of the *Salmonella* Paratyphi B *d*Ta+ isolate 08-00436 (GenBank accession no. CP020492) to filter out reads representing the bacterial chromosome. Unmapped reads were subjected to *de novo* assembly followed by *RASTtk* annotation to identify putative novel *mcr* genes. For strain 13-SA01718 a novel plasmid-encoded phosphoethanolamine transferase gene (later termed as *mcr-5*) was identified. The respective plasmid sequence pSE13-SA01718 (12 201 bp) was obtained by *de novo* assembly of trimmed but unfiltered sequence data. To investigate the prevalence of the *mcr-5* gene in our strain selection and to confirm its ability to mediate colistin resistance, the following studies were carried out.

Retrospective screening for mcr-5 in the Salmonella Paratyphi B dTa+ strain collection

PCR screening on the presence of *mcr-5* was performed with thermal cell lysis preparations obtained as previously described.[21] PCR amplifications were prepared in 25 μL reactions containing 2.5 μL of 10× PCR Rxn Buffer, 0.75 μL of 50 mM $MgCl_2$, 2.5 μL of 200 μM dNTP Mix (prepared from 100 mM dNTP solutions), 0.2 μL of Platinum® Taq DNA Polymerase (Invitrogen), 2.5 μL of 10 μM primer dilutions (PCR primers: MCR5_fw, 5′-ATGCGGT TGTCTGCATTTATC-3′; and MCR5_rev, 5′-TCATTGTGGTTGTCCTTTTCTG-3′; Eurogentec, Seraing, Belgium), 12.05 μL of sterile water and 2 μL of template DNA. The PCR conditions were chosen as follows: 5 min 95 °C initial denaturation, 30 cycles of 30 s 95 °C denaturation, 30 s 50 °C primer annealing and 95 s 72 °C elongation followed by 5 min 72 °C final elongation. Positive isolates showing a PCR product of 1644 bp were subjected to S1-PFGE analysis and WGS.

Determination of mcr-5 location of identified isolates by S1-PFGE and DNA-DNA hybridization

Plasmid profiles of *mcr-5*-harbouring isolates were analysed by S1-PFGE, Southern blotting and DNA-DNA hybridization experiments as previously described[22] using a digoxigenin-11-dUTP labelled *mcr-5* probe. The probe (613 bp) was prepared by PCR amplification (PCR primers: MCR5-intern_fw: 5′-TATCTCGACAAGGCCATGCTG-3′; MCR5-intern_rev: 5′-GAATCTGGCGTTCG TCGTAGT-3′) using the above-mentioned protocol and exchanging the dNTP solution with PCR DIG Labelling Mix (Roche Applied Science, Mannheim, Germany). PCR conditions were adjusted by choosing an annealing temperature of 54 °C and an elongation time of 40 s. Following the PCR the probe was purified by gel extraction using the GFX™ DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK).

Investigation of further identified mcr-5-carrying isolates by WGS

To analyse the similarity of *mcr-5*-harbouring plasmids to the initially identified plasmid pSE13-SA01718, obtained raw reads from the 12 isolates carrying *mcr-5* plasmids similar to 13-SA01718 were trimmed and mapped against the pSE13-SA01718 sequence using CLC Genomics Workbench. In the case of isolate 12-02541, which showed an increased plasmid size by S1-PFGE, *de novo* assembly was carried out to determine the plasmid sequence of pSE12-02541 (17 156 bp) as described above. Plasmid sequences were compared by BLASTn alignment and visualized using BRIG.[23] The size-differentiating plasmids pSE13-SA01718 and pSE12-02541 were further subjected to transformation experiments. In the case of subclone 12-02546-2, which did not show any *mcr-5* plasmid, trimming and *de novo* assembly were carried out to generate a draft genome sequence represented in contigs. Whole-genome SNP analysis was performed with the

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help of BioNumerics 7.6 (Applied Maths, Gent, Belgium) using the chromosome sequence of isolate 08-00436 as reference. Based on the resulting SNP matrix the pairwise distances between the isolates were calculated and clustering of isolates was performed using the Neighbour Joining algorithm.

Determination of mcr-5 copy numbers

To estimate the copy number of *mcr-5*, trimmed sequencing reads of selected isolates were mapped against both, *mcr-5* and the chromosomally located house-keeping gene *aroC* (MLST allele aroc-15 provided by the CGE MLST tool; https://cge.cbs.dtu.dk/services/MLST/). The *mcr-5*/*aroC* ratio was determined based on the average per base sequence coverage of the two genes. The *mcr-5* copy number per cell was calculated assuming that each cell harbours only one copy of the bacterial chromosome as described previously.[24]

Transformation of mcr-5 plasmids from Salmonella Paratyphi B dTa+ into E. coli DH5α

Plasmid DNA was extracted following the protocol from Birnboim and Doly[25] and transferred into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) following the manufacturer's instructions. Transformants were selected on LB agar pre-incubated with a final concentration of 2 mg/L colistin sulphate (pSE13-SA01718) or supplemented with 100 mg/L ampicillin (pSE12-02541, the co-selection is possible due to the additional presence of a bla_{TEM} resistance cassette on the plasmid). Transformants were confirmed by *mcr-5* PCR and subjected to WGS and antimicrobial susceptibility testing.

Cloning of the mcr-5 operon in pCR2.1 and transformation of a Salmonella Paratyphi B dTa+ control strain

The *mcr-5*-carrying operon (3683 bp) including the ChrB domain protein, *mcr-5*, ORF1 and ORF2 as well as upstream and downstream elements was amplified by PCR (PCR primers: MCR5-Operon_fw 5′-GGTATGCCTCAAC CGGAC-3′; MCR5-Operon_rev 5′-CGCTGCTAGGGGTGTCT-3′) using the above-mentioned protocol and adjusting PCR conditions as follows: annealing temperature at 57 °C and elongation time at 220 s. The PCR product was ligated into the pCR™ 2.1-TOPO® vector (TOPO® TA Cloning® Kit; Invitrogen) and the obtained vector construct pCR2.1-MO was transferred in One Shot® chemically competent *E. coli* TOP10F′. Transformants were selected on LB agar supplemented with 100 mg/L ampicillin using the vector-encoded ampicillin resistance marker. Suspected positive colonies were confirmed by *mcr-5* operon PCR and plasmid DNA was isolated using the Invisorb® Spin Plasmid Mini Two Kit (Stratec, Berlin, Germany) and subjected to Sanger sequencing (Eurofins Genomics, Ebersberg, Germany) to confirm the insert sequence. Chemically competent cells of *Salmonella* Paratyphi B *d*Ta+ control strain 13-SA01617 with a WT phenotype for colistin were prepared using the following protocol. An overnight culture was diluted 1:100 in LB and incubated at 37 °C with gentle shaking until an OD_{600} 0.30–0.40 was reached. A 2 mL aliquot of the culture was centrifuged and harvested at 4 °C and 10000 **g** for 10 min. The supernatant was discarded and cells were resuspended in 50 μL of 100 mM $CaCl_2$ solution. A 25 μL aliquot of cells was mixed with 2 μL of pCR2.1-MO plasmid DNA (~30 ng) and incubated on ice for 30 min. Heat shock was performed for 2 min at 42 °C. A 975 μL aliquot of LB was added and cells were incubated for 1 h at 37 °C with gentle shaking. The transformation mix was plated on LB agar supplemented with ampicillin (100 mg/L) and incubated overnight at 37 °C. Positive colonies were analysed by PCR and subjected to antimicrobial susceptibility testing and WGS.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using broth microdilution following CLSI guidelines (CLSI M07-A9) and EUCAST epidemiological cut-off values (ECOFFs; http://www.eucast.org/). According to EUCAST ECOFFs, *Salmonella* and *E. coli* isolates are defined as having a non-WT phenotype for colistin when showing an MIC >2 mg/L.[26]

Nucleotide sequence accession numbers

The plasmid sequences pSE13-SA01718 (GenBank accession no. KY807921) and pSE12-02541 (GenBank accession no. KY807920), the *de novo* assembly for strain 12-02546-2 (BioSample accession no. SAMN06627639) as well as raw read data of the *mcr-5*-positive *Salmonella* isolates (BioProject accession no. PRJNA396070) were deposited in the NCBI database.

Results and discussion

A novel transposon-associated phosphoethanolamine transferase gene (mcr-5) was identified in 14 Salmonella Paratyphi B dTa+ isolates collected in 2011–13

Using a WGS analysis approach, we identified a novel phosphoethanolamine transferase gene in *Salmonella* Paratyphi B *d*Ta+ isolate 13-SA01718, which showed a non-WT phenotype for ampicillin, ciprofloxacin, colistin, nalidixic acid, sulfamethoxazole, tetracycline and trimethoprim. The respective gene (1644 bp), further termed as *mcr-5*, was located on a 7337 bp Tn*3*-family transposon harboured by a 12201 bp ColE-type plasmid designated as pSE13-SA01718 (Figures 1 and 2a).

Following these findings 31 additional *mcr-1*-, *mcr-2*- and *mcr-3*-negative *Salmonella* Paratyphi B *d*Ta+ isolates (with colistin MIC values >2 mg/L) received in the years 2011–16 and originating from food-producing animals and food products were selected for PCR screening. Altogether 11 strains with a similar phenotypic pattern as 13-SA01718 as well as two additional strains (non-WT phenotype for ciprofloxacin, colistin, nalidixic acid and trimethoprim) were found to be positive for *mcr-5* (Table 1). All 14 *mcr-5*-positive isolates were analysed by S1-PFGE, Southern blotting, DNA-DNA hybridization and WGS. Evaluation of S1-PFGE experiments showed that 12 isolates harboured *mcr-5*-carrying plasmids with the same size as pSE13-SA01718 (Figure 3). Sequence data obtained for these isolates were mapped to the sequence of plasmid pSE13-SA01718. The results revealed that all isolates contain a plasmid with 100% identity to pSE13-SA01718. For isolate 12-02541 S1-PFGE results indicated the presence of an *mcr-5*-harbouring plasmid with increased molecular size compared with pSE13-SA01718. *De novo* assembly of the respective sequence data showed that the isolate harboured a highly related 17156 bp ColE-type plasmid designated as pSE12-02541 containing an additional bla_{TEM-1b} gene on a Tn*3*-type transposon (Figure 1). Interestingly, a subclone of isolate 12-02546 (12-02546-2), which most likely evolved during laboratory culturing, did not show an *mcr-5*-harbouring plasmid (Figure 3). Bioinformatic analysis of the *de novo* assembled sequence data led to the finding that the 7337 bp *mcr-5* transposon has integrated in an ornithine decarboxylase gene within the bacterial chromosome (contig 17).

Whole-genome SNP analysis revealed that all isolates from 2012 were highly similar (0–6 SNPs). Based on the fact that these

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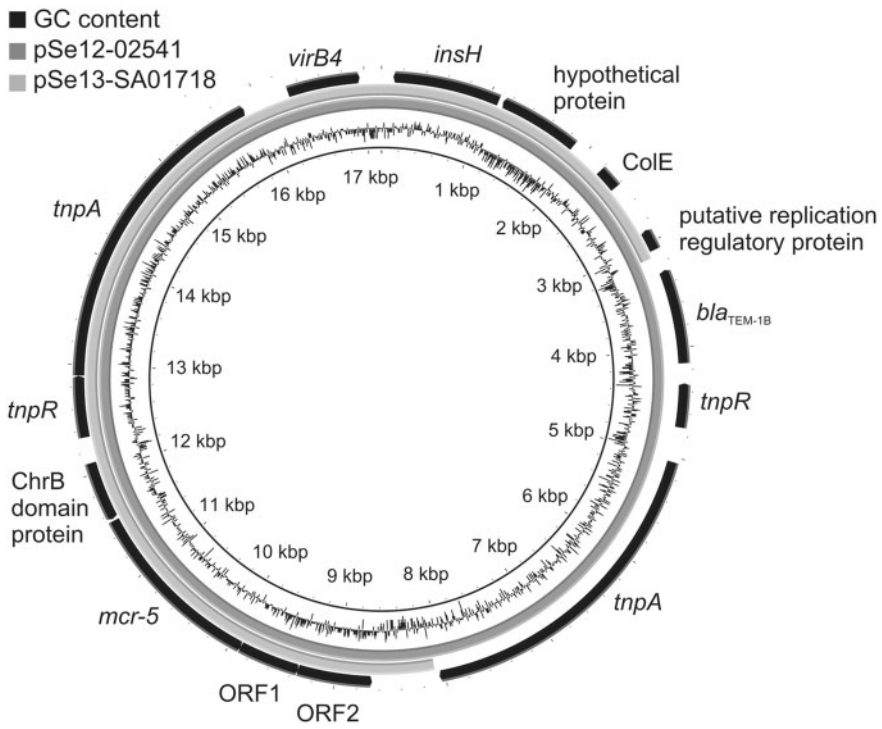

Figure 1. Structure of *mcr-5*-harbouring plasmids pSE13-SA01718 (GenBank accession no. KY807921) and pSE12-02541 (GenBank accession no. KY807920) isolated from *Salmonella* Paratyphi B *d*Ta+ isolates 13-SA01718 and 12-02541.

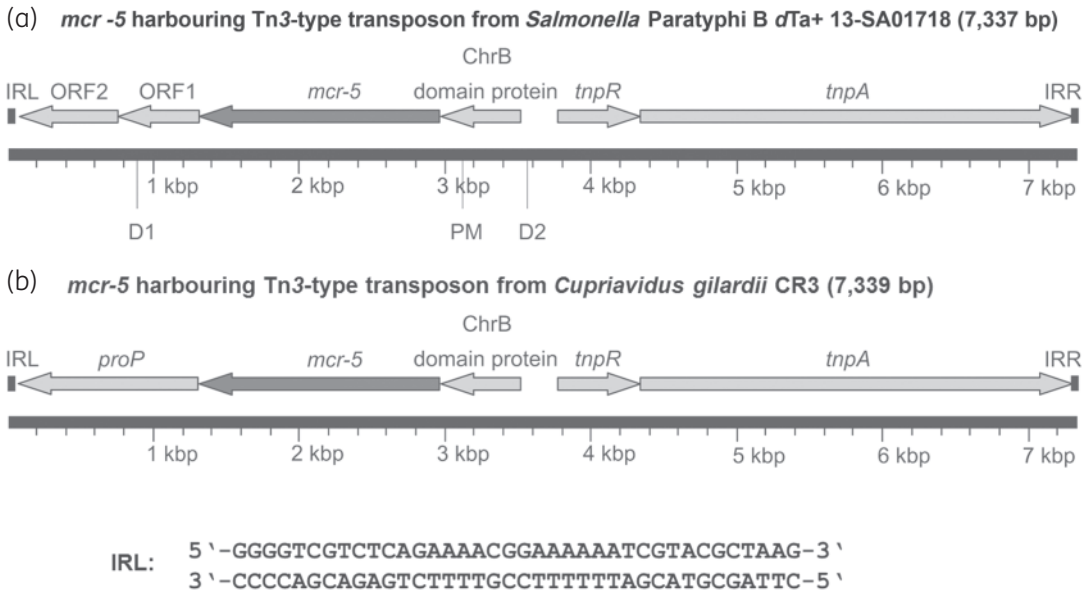

Figure 2. Comparative schematic genetic organization of *mcr-5*-harbouring transposons from *Salmonella* Paratyphi B *d*Ta+ and *C. gilardii* CR3 (GenBank accession no. CP010516.1). Lengths of transposons are illustrated in kbp. D1, 1 bp deletion in the *proP* ORF; D2, 1 bp deletion in an untranslated region; PM, point mutation leading to the amino acid substitution ChrB Q134L; IRL/IRR, 38 bp IRL/IRR.

strains (as far as known) were isolated in the same German state, a clonal expansion is very likely. However, the two other isolates from 2011 and 2013 differ from these isolates not only in the state of isolation but also in 16–20 SNPs [Table 1 and Figure S1 (available as Supplementary data at *JAC* Online)].

The mcr-5 gene is encoded in an operon located on a Tn3-family transposon that is also present in Cupriavidus gilardii

Determination of the genetic background of *mcr-5* revealed that the gene is likely to be encoded in an operon with a ChrB domain

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Table 1. Overview of *mcr-5*-carrying *Salmonella* Paratyphi B *d*Ta+ isolates, transformants and negative control strains

Isolate name	Year of isolation	Place of isolation	Matrix	Non-WT phenotype for	Colistin MIC (mg/L)	*mcr-5* location
11-00422	2011	Berlin	chicken meat	AMP, CIP, **CST**, NAL, SMX, TET, TGC, TMP	8	pSE13-SA01718
12-00618	2012	Lower Saxony	poultry	CIP, **CST**, NAL, TMP	8	pSE13-SA01718
12-00619	2012	Lower Saxony	poultry	AMP, CIP, **CST**, NAL, SMX, TET, TMP	8	pSE13-SA01718
12-00663	2012	Lower Saxony	environment	AMP, CIP, **CST**, NAL, SMX, TET, TMP	8	pSE13-SA01718
12-01497	2012	Lower Saxony	poultry	AMP, CIP, **CST**, NAL, SMX, TET, TMP	8	pSE13-SA01718
12-01549	2012	Lower Saxony	poultry	AMP, CIP, **CST**, NAL, SMX, TET, TGC, TMP	8	pSE13-SA01718
12-02539	2012	Lower Saxony	bacterial culture	CIP, **CST**, NAL, TMP	8	pSE13-SA01718
12-02541	2012	Lower Saxony	poultry	AMP, CIP, **CST**, NAL, SMX, TET, TMP	8[a]	pSE12-02541
12-02544	2012	Lower Saxony	poultry	AMP, CIP, **CST**, NAL, SMX, TET, TGC, TMP	8	pSE13-SA01718
12-02546-1	2012	Lower Saxony	environment	AMP, CIP, **CST**, NAL, SMX, TET, TGC, TMP	8	pSE13-SA01718
12-02546-2	2012	Lower Saxony	environment	AMP, CIP, **CST**, NAL, SMX, TET, TMP	4[a]	chromosome
12-02548	2012	Lower Saxony	poultry	AMP, CIP, **CST**, NAL, SMX, TET, TGC, TMP	8	pSE13-SA01718
12-02552	2012	Lower Saxony	poultry	AMP, CIP, **CST**, NAL, SMX, TET, TGC, TMP	8	pSE13-SA01718
12-03724	2012	unknown	chicken meat	AMP, CIP, **CST**, NAL, SMX, TET, TMP	8	pSE13-SA01718
13-SA01718	2013	Mecklenburg-West Pomerania	chicken meat	AMP, CIP, **CST**, NAL, SMX, TET, TMP	8[a]	pSE13-SA01718
DH5α (negative control strain)	–		–	NAL	≤1	–
DH5α pSE13-SA01718	–		–	**CST**, NAL	8	pSE13-SA01718
DH5α pSE12-02541	–		–	AMP, **CST**, NAL	4	pSE12-02541
13-SA01617 (negative control strain)	2013	North Rhine-Westphalia	chicken meat	CIP, NAL, TMP	2	–
13-SA01617 pCR2.1-MO	–		–	AMP, CIP, **CST**, NAL, TMP	8	pCR2.1-MO

AMP, ampicillin; CIP, ciprofloxacin; CST, colistin; NAL, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; TGC, tigecycline.
[a]MIC values of colistin were determined in six independent experiments leading to the same results.

protein involved in chromate resistance[27,28] and two ORFs encoding protein fragments *RASTtk*-classified as uncharacterized major facilitator superfamily (MFS)-type transporter. The complete operon is located on a 7337 bp Tn*3*-type transposon flanked by IRs (38 bp) (Figure 2a). A BLASTn search revealed that this transposon is also present in chromosome 1 of *C. gilardii* strain CR3 isolated in the USA (GenBank accession no. CP010516.1; Figure 2b). *C. gilardii* is known as an aerobic, Gram-negative bacterium that can be isolated from a broad spectrum of environmental sources and is rarely associated with human infections.[29–31] The transposon from *Salmonella* Paratyphi B *d*Ta+ differs from the *C. gilardii* transposon by the absence of two single nucleotides and a point mutation. Deletion 1 regards an ORF encoding the MFS-type proline/betaine transporter ProP leading to frameshift and ultimately resulting in the MFS-type transporter gene fragments found in the *Salmonella* Paratyphi B *d*Ta+ transposon. Deletion 2 is located in an untranslated region and the point mutation was identified in the ChrB domain protein leading to the amino acid exchange ChrB Q134L (Figure 2).

Cloning and transformation experiments revealed that mcr-5 leads to increased colistin MIC values for E. coli DH5α and Salmonella Paratyphi B dTa+ control strains

The capability of *mcr-5* to cause colistin resistance (MIC >2 mg/L) was proven in two different experiment settings. First, *mcr-5*-carrying plasmids pSE13-SA01718 and pSE12-02541 were transferred in *E. coli* DH5α. Positive transformants DH5α pSE13-SA01718 and DH5α pSE12-02541 were subjected to WGS to verify plasmid sequences. MIC testing using broth microdilution revealed MIC values

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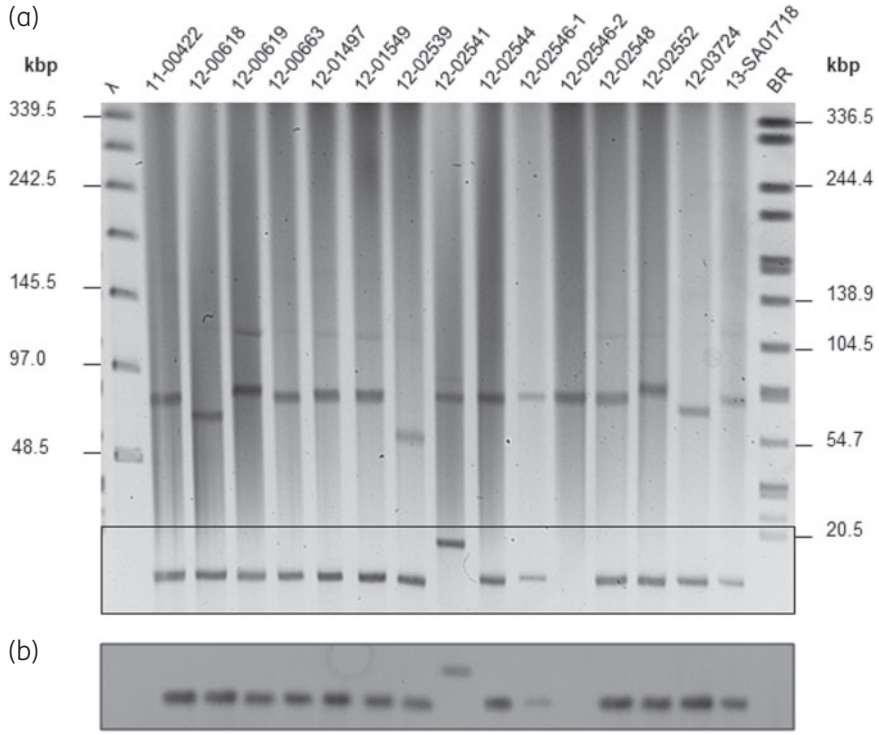

Figure 3. S1-PFGE, Southern blot and hybridization analysis of *mcr-5*-harbouring *Salmonella* Paratyphi B *d*Ta+ isolates. (a) S1-PFGE analysis of *mcr-5*-positive *Salmonella* Paratyphi B *d*Ta+ isolates. (b) Southern blot and hybridization analysis using an *mcr-5* probe. BR, *Salmonella* Braenderup H9812 restricted with XbaI; λ, CHEF DNA Size Standard, Lambda Ladder (Bio-Rad Laboratories, Hercules, CA, USA).

of 8 and 4 mg/L, respectively (Table 1). In the second setting, the whole *mcr-5* operon including the ChrB domain protein, *mcr-5*, ORF1 and ORF2 as well as upstream and downstream elements was ligated into the pCR2.1 vector to generate plasmid pCR2.1-MO, which was finally transformed in *Salmonella* Paratyphi B *d*Ta+ isolate 13-SA01617 with a WT phenotype for colistin (MIC = 2 mg/L). The transformant 13-SA01617 pCR2.1-MO showed a colistin MIC value of 8 mg/L (Table 1).

Copy number of mcr-5 effects MIC values for Salmonella Paratyphi B dTa+

With the help of *in silico* typing using previously published primer sequences (oricolE FW and oricolE RV)[32] and taking into consideration two mismatches in oricolE FW (resulting in the primer 5′-GTTCGTGCACACAGCCCA-3′) the plasmids pSE13-SA01718 and pSE12-02541 could be allocated to the multi-copy ColE-type plasmid group. These plasmids were previously reported to be involved in antimicrobial resistance by harbouring genes mediating resistance to quinolones, aminoglycosides and extended-spectrum β-lactams.[32–35]

The copy number for *mcr-5* located on multi-copy ColE-type plasmids varies ∼27–188 with an average at 68 copies per cell. These numbers represent snapshots and are likely influenced by the cultivation conditions, intensity of antibiotic selection pressure, state of the cells at the time of the DNA extraction, method used for DNA extraction as well as the library preparation and the sequencing process itself.[24,36,37] Nevertheless the observed numbers are in concordance with studies describing ColE-type plasmids in Gammaproteobacteria as small multi-copy plasmids with copy numbers as high as 50 or even higher.[24,38]

When comparing the MIC values of colistin for our *mcr-5*-positive *Salmonella* Paratyphi B *d*Ta+ isolates, MIC values of 8 mg/L were observed for all isolates carrying the *mcr-5* transposon on multi-copy ColE-type plasmids. Only the variant 12-02546-2 with theoretically only one *mcr-5* copy located on the chromosome (calculated *mcr-5* copy number per cell: 1.53) showed an MIC of 4 mg/L (Table 1). These results indicate a higher degree of antimicrobial resistance due to copy number effects. Enhanced antibiotic resistance in Gammaproteobacteria mediated by increased copy numbers of small ColE plasmids was also previously described by San Millan *et al.*[36]

The MCR-5 protein sequence is distinct from MCR-1, MCR-2, MCR-3 and MCR-4 and of unknown origin

The *mcr-5* gene encodes a 547 amino acid protein annotated as phosphoethanolamine transferase using *RASTtk*. The MCR-5 protein domain structure was predicted using the Simple Modular Architecture Research Tool (SMART)[39,40] and the MCR-5 protein sequence was aligned to MCR-1 (GenBank accession no. WP_049589868.1), MCR-2 (GenBank accession no. WP_065419574.1), MCR-3 (GenBank accession no. WP_039026394.1) and MCR-4 (GenBank accession no. ASR73329.1) using Clustal Omega.[41] Results show that MCR-5 can be divided into three domains: a transmembrane domain, a domain of unknown function

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(DUF1705) and a sulphatase domain, which are also observed for MCR-1, MCR-2, MCR-3 and MCR-4 (Figure S2). Compared with MCR-1 and MCR-2, which show an amino acid sequence identity of 81.23% and are both believed to originate from *Moraxella* spp.,[14,42] MCR-5 is distinct from MCR-1 and MCR-2 with a protein sequence identity of 36.11% (MCR-1) and 35.29% (MCR-2). With protein sequence identities of 34.72% and 33.71%, MCR-5 is also distinct from MCR-3 and MCR-4, which align closely to phosphoethanolamine transferases from *Aeromonas* spp. and *Shewanella frigidimarina*, respectively.[15,16] Nevertheless conserved residues can be found in the C-terminal sulphatase domain including the five MCR-5 residues (E248, T286, H389, D458 and H459), which can be aligned to identical residues in MCR-1, MCR-2, MCR-3 and MCR-4 (Figure S2). These residues were hypothesized to be critical for substrate binding and MCR-1-mediated colistin resistance.[43]

BLASTp search of MCR-5 identified hits to phosphoethanolamine transferases from Proteobacteria (GenBank accession no. WP_053821788.1, 100% identity and 100% query coverage) not only annotated in *C. gilardii* CR3 from the USA (GenBank accession no. ALD90568.1), but also in *Salmonella enterica* subsp. *enterica* serovar Schwarzengrund from France (GenBank accession no. KZB53132.1) and *Pigmentiphaga* sp. from Canada (GenBank accession no. OVZ64486.1). A large part of MCR-5 (amino acids 1–404) was also found to be identical to parts of a hypothetical protein from *Pseudomonas aeruginosa* from Japan (WP_079452551, 100% identity and 73% query coverage). Further analysis of the underlying assemblies of *Salmonella* Schwarzengrund (GenBank accession no. GCA_001614105.1), *Pigmentiphaga* sp. (GenBank accession no. GCA_002188465.1) and *P. aeruginosa* (GenBank accession no. GCA_000974565.1) revealed that all of them carried parts of the *mcr-5* transposon (Figure S3). However, these findings should be viewed critically given that read contamination as well as assembly errors cannot be excluded based on the available assembly data. Further BLASTp hits to MCR-5 are more distant ($\leq$53% identity and $\leq$96% query coverage) and can be found in a broad range of Proteobacteria. Therefore, the origin of *mcr-5* remains unknown from today's point of view.

Challenges in naming our novel colistin resistance genes

Naming of the novel phosphoethanolamine transferase gene proved to be challenging. Previously described *mcr* genes were either located on conjugational IncI2, IncX4, IncHI1B, IncHI2A, IncFII or IncFIB plasmids (*mcr-1*, *mcr-2* and *mcr-3*)[9,14,15,43] or on ColE-type plasmids mobilizable by a helper plasmid (*mcr-4*).[16] Our *mcr-5* gene is harboured by small multi-copy ColE-type plasmids that do not carry any transfer genes involved in plasmid conjugation. However, transposition of the *mcr-5* transposon as observed *in vitro* for the isolate subvariant 12-02546-2 has the potential to occur *in vivo* leading to a transfer of the transposon to conjugational plasmids. The possibility of an intra- or even inter-species transfer of the *mcr-5*-carrying transposon is supported by the presence of this genetic element in *C. gilardii*. Therefore, we decided to follow Liu *et al.*,[9] Xavier *et al.*,[14] Yin *et al.*[15] and Carattoli *et al.*[16] and name the newly discovered colistin resistance gene *mcr-5*.

Conclusions

In this study, we reported a new transposon-associated phosphoethanolamine transferase mediating colistin resistance in *Salmonella* Paratyphi B *d*Ta+. In our strain collection, *mcr-5* is restricted to a small number of *Salmonella* Paratyphi B *d*Ta+ isolates received during 2011–13. However, the presence of the *mcr-5* transposon or parts thereof in other Proteobacteria including *C. gilardii*, *Salmonella* Schwarzengrund, *Pigmentiphaga* sp. and *P. aeruginosa* isolated from different places around the world questions the real prevalence of *mcr-5* in Gram-negative bacteria. Moreover, the detection of a further *mcr* gene suggests that the transfer of colistin-resistance-mediating genes from bacterial chromosomes to mobile elements might have occurred in at least five independent events raising concern regarding the real variety of mobilizable colistin resistance genes as well as their prevalence and importance in public health. Therefore, these findings call for extended screening of known and further exploration of unknown colistin resistance mechanisms in Gram-negative pathogens as well as in commensals.

Acknowledgements

We gratefully thank Martha Brom, Ernst Junker and Beatrice Baumann for excellent laboratory assistance and Beatriz Guerra for critical reading of the manuscript prior to submission.

Funding

This work was supported by the German Federal Institute for Risk Assessment (BfR, 1322-639) and the project 'Establishing Next Generation sequencing Ability for Genomic analysis in Europe' (ENGAGE) funded by the European Food Safety Authority (EFSA, GP/EFSA/AFSCO/2015/01/CT1).

Transparency declarations

None to declare.

Disclaimer

The conclusions, findings and opinions expressed in this scientific paper reflect only the view of the authors and not the official position of the European Food Safety Authority.

Supplementary data

Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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