Characterization of a novel macrolide efflux gene, mef(B), found linked to sul3 in porcine Escherichia coli

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Objectives: The aim of this study was to characterize a putative novel macrolide efflux gene located in the vicinity of sul3 in porcine Escherichia coli.

Methods: Five sul3-encoding E. coli isolates of porcine origin were investigated by plasmid characterization and random amplification of polymorphic DNA (RAPD) PCR. Unknown DNA adjacent to the sul3 genes was amplified using a PCR approach, followed by sequencing of the fragments. The putative macrolide efflux gene was cloned into pK18. The cloned gene was characterized by susceptibility testing by Etest in the presence and absence of efflux inhibitors.

Results: Five sul3-encoding isolates, demonstrated to be unrelated by RAPD PCR, were characterized. The immediate genetic context of sul3 in five isolates was identical to that in plasmid pVP440, and in all cases, sul3 was associated with class 1 integrons. In three isolates, an open reading frame (orf2) encoding a putative protein with 38% amino acid identity to Mef(A) was found, while the two remaining isolates contained a fragment of orf2 truncated by IS26 insertion. In three of the isolates, this DNA region was demonstrated to be located on non-conjugative plasmids. When the complete orf2 was cloned, it conferred high-level resistance to erythromycin and azithromycin, and the resistance property could be partially inhibited using the efflux inhibitor Phe-Arg β-naphthylamide dihydrochloride. The gene was named mef(B).

Conclusions: A new macrolide efflux protein, Mef(B), with 38% amino acid identity to Mef(A), has been characterized and represents the second member of the mef family of genes.

Keywords: macrolide resistance, mef(A), plasmids, integrons

Introduction

Sulphonamides were the first group of systemic antimicrobials introduced into medicine and have been used extensively since then.1 For decades, only two acquired sulphonamide resistance genes were known, sul1 and sul2, both of which encode plasmid-borne sulphonamide-resistant dihydropteroate synthase enzymes.1 However, a third sulphonamide resistance gene, sul3, was described in 2003, isolated from porcine Escherichia coli.2 Emergence of sul3 implies that there is still positive selection pressure for sulphonamide resistance, despite the use of the drug being limited in many countries. This may due to the relatively high use of trimethoprim/sulphamethoxazole in veterinary medicine, along with other antimicrobials such as tetracyclines and macrolides.

The mef(A) gene encodes a macrolide-efflux pump that mediates resistance to 14- and 15-membered macrolides in Gram-positive bacteria.3 It belongs to the major facilitator superfamily (MFS) of efflux pumps and is encoded on transposable elements such as Tn1207.1,3 Although enteric bacteria such as E. coli are often non-susceptible to macrolide antibiotics due to the presence of chromosomal efflux pumps, they can nevertheless acquire genes such as mef(A) or erm(B) that increase their resistance levels further.3

While investigating the prevalence and genetic context of sul3 among porcine E. coli from Great Britain, a novel gene coding for a putative macrolide efflux protein was identified upstream of sul3 in three isolates, the characterization of which is described here.

Materials and methods

Bacterial isolates and MIC determination

Sixty-two sulphonamide-resistant E. coli obtained from a study investigating the prevalence of antimicrobial resistance genes among
isolates from farm animals were studied.5 Susceptibility to ampicillin, azithromycin, chloramphenicol, clindamycin, erythromycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim was determined by Etest (AB Biodisk, Solna, Sweden), and susceptibility to ciprofloxacin, ceftazidime, gentamicin, kanamycin and nalidixic acid was determined by disc diffusion. All susceptibility tests were carried out and interpreted according to BSAC guidelines.6

**PCR and DNA sequencing**

PCR amplification was used to detect the presence of sul3 and class 1 integron integrase, using previously published primers and conditions,2,5 with Hi-Fidelity PCR Master Mix (Thermo, Epsom, UK). The putative macrolide efflux gene was sequenced during an investigation of the genetic context of sul3, which was determined using a two-step random PCR technique1 in which primers targeted at the extremities of sul3 were used in conjunction with random primers to obtain the adjacent unknown sequence. Amplification products were separated by agarose gel electrophoresis and visualized under UV light. They were purified with the Qiaquick Gel Extraction Kit (Qiagen), according to the manufacturer’s instructions, and sent for sequencing at the Advanced Biotechnology Centre, Imperial College, London, UK. Gaps in sequence were filled by primer walking. Sequence analysis was carried out using the Lasergene DNASTAR software package. Similarity to known sequences was searched for using BLAST. The GenBank accession numbers of the sequences described here are FJ196384 (P126.10.99.C1), FJ196385 (P286.10.99.C2), FJ196386 (P328.10.99.C2), FJ196387 (P475.10.99.C3) and FJ196388 (P528.10.99.C4).

**Random amplification of polymorphic DNA (RAPD) PCR**

RAPD PCR was carried out to determine relatedness among isolates, as described previously.8

**Plasmid analysis**

Transfer of antibiotic resistance plasmids by conjugation was attempted using the agar mating method,9 using E. coli K12 JM109 as the recipient. Plasmid DNA was extracted from E. coli isolates grown overnight in Luria–Bertani (LB) broth containing 500 mg/L sulfamethoxazole, using a Qiaquick Miniprep Kit (Qiagen), according to the manufacturer’s instructions. Plasmid DNA was transformed by electroporation into electrocompetent E. coli K12 JM109 cells. Transconjugants and transformants were selected on Iso-Sensitest agar (Oxoid, Basingstoke, UK), containing 500 mg/L sulfamethoxazole, using a Qiaquick Miniprep Kit (Qiagen), according to the manufacturer’s instructions, and sent for sequencing at the Advanced Biotechnology Centre, Imperial College, London, UK. Gaps in sequence were filled by primer walking. Sequence analysis was carried out using the Lasergene DNASTAR software package. Similarity to known sequences was searched for using BLAST. The GenBank accession numbers of the sequences described here are FJ196384 (P126.10.99.C1), FJ196385 (P286.10.99.C2), FJ196386 (P328.10.99.C2), FJ196387 (P475.10.99.C3) and FJ196388 (P528.10.99.C4).

**Cloning**

To obtain the DNA sequence of the antibiotic resistance-encoding region of pP286, plasmid DNA was digested with BamHI and HindIII or EcoRI and PstI (Promega). Digested DNA was ligated into pK18 vector and transformed into E. coli K12 JM109. Transformants were selected on LB agar containing kanamycin (30 mg/L), X-gal (40 mg/L) and IPTG (1 mM). Plasmid DNA was extracted from white colonies and the DNA sequence of the inserts obtained, as described earlier. To clone the putative macrolide efflux gene on pP286, DNA was digested with PstI and SacI, ligated into pK18 and transformed by electroporation into E. coli K12 JM109.

**Table 1. Characteristics of five E. coli isolates encoding complete or truncated mef(8) genes and of E. coli, K12 JM109 with and without plasmid pK18mef encoding the cloned mef(8) gene**

<table>
<thead>
<tr>
<th>E. coli isolate</th>
<th>Resistance profile</th>
<th>Resistance genotype</th>
<th>Resistance profile</th>
<th>Resistance genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>P126.10.99.C1</td>
<td>AZM, CHL, CLI, FRY</td>
<td>sul3, dhfr12, mef(B)</td>
<td>P286.10.99.C2</td>
<td>CHL, STR, SMX, TET</td>
</tr>
<tr>
<td>P328.10.99.C2</td>
<td>AZM, CHL, FRY</td>
<td>sul3, dhfr12, mef(B)</td>
<td>P475.10.99.C3</td>
<td>CHL, STR, SMX, TET</td>
</tr>
<tr>
<td>P475.10.99.C3</td>
<td>AZM, ERY</td>
<td>sul3, mef(B)</td>
<td>P528.10.99.C4</td>
<td>CHL, STR, SMX, TET</td>
</tr>
<tr>
<td>K12 JM109</td>
<td></td>
<td>mef(B)</td>
<td>K12 JM109 pK18mef</td>
<td>AZM, CLI, ERY</td>
</tr>
<tr>
<td>K12 JM109</td>
<td></td>
<td>mef(B)</td>
<td>AMP, ampicillin</td>
<td>AZM, chloramphenicol, CLI, clindamycin, GEN, gentamicin, FRY, erythromycin, STR, streptomycin, SMX, sulfamethoxazole, TET, tetracycline, TAP, trimethoprim</td>
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<tr>
<th>E. coli K12 JM109</th>
<th>Resistance profile</th>
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<td>mef(B)</td>
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K12 JM109. Transformants were selected on LB agar containing kanamycin and erythromycin (250 mg/L). The presence of the macrolide efflux gene in the clones was verified by PCR using the primers MEFBF 5'-ATGAACAGAATAAAAAATTG-3' and MEFBR 5'-AAATTATCATCAACCCGGTC-3' at an annealing temperature of 45°C using standard protocols, as described earlier. One gene-positive clone of the expected size was chosen for further studies and named pK18mef.

Inhibitor studies of cloned putative macrolide efflux gene

The susceptibility of E. coli K12 JM109 with and without the pK18mef clone to erythromycin was measured by Etest in the presence and absence of the efflux inhibitors carbonyl cyanide m-chlorophenylhydrazone (CCCP; 1 mg/L), omeprazole (100 mg/L), Phe-Arg β-naphthylamide dihydrochloride (PAβND; 80 mg/L) and reserpine (25 mg/L). All inhibitor compounds were purchased from Sigma (Poole, UK).

Results and discussion

Characteristics of isolates and plasmids

Sixty-two sulphonamide-resistant E. coli, isolated at slaughter from healthy farm animals in Great Britain in 1999, were screened for the presence of sul3 by PCR. The antimicrobial susceptibility profile of the isolates and carriage of other resistance genes had previously been determined. Twelve isolates carried sul3, of which five were chosen for further characterization (Table 1). All five were isolated from pigs and originated from different abattoirs. They were demonstrated to be distinct by RAPD-PCR. To the best of our knowledge, these isolates are among the earliest described sul3-encoding isolates, suggesting that the sul3 gene emerged in the E. coli population during the 1990s or before.

The plasmid content of the five isolates was investigated. None of the sul3-coding plasmids could be conjugated, although they could be mobilized by larger plasmids from two of the isolates, P126.11.99.C1 and P328.10.99.C2. Transformation of sul3-coding plasmids into E. coli K12 JM109 by electroporation was attempted. Transformants were successfully obtained with plasmid preparations from three isolates: P126.11.99.C1, P286.10.99.C3 and P328.10.99.C2. The plasmids from isolates P126.11.99.C1 (pP126), P286.10.99.C3 (pP286) and P328.10.99.C2 (pP328) were estimated to be 20.2, 16.6 and 22.0 kb, respectively, in size. All transformants acquired resistance to chloramphenicol, streptomycin and sulfamethoxazole. In addition, the transformants carrying pP286 and pP126 acquired resistance to erythromycin, and the transformant carrying pP126 acquired trimethoprim resistance.

DNA sequences in the vicinity of the sul3 gene

All five isolates had sul3 sequences identical to that found on plasmid pVP440, and in all isolates, the sul3 genes were flanked by a transposase 3’ of the gene and a 752 bp non-coding region followed by an open reading frame (orf1) with homology to oxidoreductase enzymes 5′ of the sul3 gene. The isolates demonstrated genetic organization related to, but distinct from, each other, except for P286.10.99.C3 and P475.10.99.C3, which were identical (Figure 1). All five isolates carried integron gene cassettes (aadA1, qacH and aadA3) 3′ of the transposase gene and had class 1 integrase. However, no evidence of a class 1 integron 3′ conserved region was detected, with class 1 integron sequences being interrupted by the transposase gene. In three of the isolates, P286.10.99.C3, P475.10.99.C3 and P126.10.99.C1, a gene upstream of orf1 was detected. Its sequence was obtained from isolate P286.10.99.C3 and revealed the presence of a 1230 bp open reading frame (orf2), which displayed no significant similarity to DNA currently deposited in the databases, with the exception of three entries displaying a truncated version of orf2 in the same genetic context. The other two isolates, P328.10.99.C2 and P528.10.99.C4, carried a 254 bp fragment of orf2 that had been truncated by IS26 insertion.

Figure 1. (a) Schematic representation of sul3-coding region from E. coli isolates P528.10.99.C4, P328.10.99.C2 and P126.11.99.C1. Direction of arrows indicates direction of transcription. (b) Antibiotic resistance-encoding region of P286.10.99.C3. Isolates P286.10.99.C3 and P475.10.99.C3 were found to have identical sul3-coding regions. However, the sequence of P475.10.99.C3 was only obtained between the aadA1 and mef(B) genes.
Characteristics of mef(B)

The predicted product of orf2 encodes 409 amino acids and exhibits 62% protein similarity (38% protein identity) to Mef(A) from Streptococcus pneumoniae. The transformant of E. coli JM109 carrying the pP286 plasmid had acquired resistance to erythromycin. To investigate whether pP286 carried another gene that could account for the erythromycin resistance, the DNA sequence of the entire class 1 integron from pP286 was obtained, as well as that of the region immediately upstream of orf2 (Figure 1b). However, no determinants that could account for erythromycin resistance were identified.

The mef(A) gene encodes a macrolide-efflux pump belonging to the MFS family and is found to be distributed mainly among Gram-positive bacteria, but has also been reported in Gram-negative bacteria. A restriction fragment containing orf2 was cloned into pK18 and the resultant clone (pK18mef) transformed into E. coli JM109. Transformation of JM109 with pK18mef increased the erythromycin and azithromycin MICs of JM109, but did not affect the clindamycin MIC (Table 1). The susceptibility of JM109 pK18mef did not change with respect to other antimicrobials tested (data not shown). The original P286.10.99.C3, P126.11.99.C1 and P475.10.99.C3 isolates that other antimicrobials tested (data not shown). The original P286.10.99.C3, P126.11.99.C1 and P475.10.99.C3 isolates that encoded complete orf2 genes were also highly resistant to erythromycin and azithromycin, while the two isolates with truncated orf2 were not resistant (Table 1). The ability of the efflux pump inhibitors to inhibit the erythromycin resistance was investigated. Reserpine, CCCP and omeprazole had no effect on the erythromycin MIC of JM109 pK18mef; however, PAβN reduced it from >256 to 12 mg/L.

The Mef(A) efflux pump confers M-type macrolide resistance, but does not confer resistance to other antimicrobials. The fact that the protein encoded by orf2 exhibits similarity to Mef(A), also confers M-type macrolide resistance and is inhibited by the efflux inhibitor PAβN strongly suggests that orf2 also encodes a macrolide efflux pump related to Mef(A). It has therefore been named Mef(B), with approval from the nomenclature centre for MLS genes.

It interesting to note that mef(B) was detected in E. coli, a species in which prevalence of macrolide non-susceptibility due to intrinsic mechanisms is high. The fact that the GC content of the gene (44.95%) differs from that of E. coli and that mef(B) is flanked by an insertion sequence (IS26) on one side and genes that also have lower GC contents (orf1 and sul3, 44.0% and 37.8%, respectively) on the other side suggests horizontal acquisition from another organism. The IS26 element flanking mef(B) may have been involved in its mobilization. The genetic location of mef(B) is considerably different from that of mef(A), which is normally found on conjugative transposons, whereas here, mef(B) was plasmid-located, implicating a role for plasmids in its transmission.

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Transparency declarations

None to declare.

References