Relevance of hot spots in the evolution and transmission of Tn1546 in glycopeptide-resistant Enterococcus faecium (GREF) from broiler origin

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Objectives: Glycopeptide-resistant enterococci are still present within the broiler sector, despite the EU ban of avoparcin more than a decade ago. In the present study, we have developed a rapid method for screening the flanking regions at the integration point of Tn1546 in glycopeptide-resistant Enterococcus faecium isolated from broiler farms.

Methods: Total DNA was digested, ligated and amplified using primers from inside Tn1546. The resulting amplicons were purified and sequenced. Two new primers were designed based on obtained sequences.

Results: Two main insertion points have been repeatedly found in isolates from the UK (n = 150). The first insertion point revealed that 25 isolates harboured Tn1546 positioned in a sequence with 96% homology to a streptomycin adenyltransferase gene (AY604739) from a Staphylococcus intermedius plasmid. At this insertion point, a direct repeat (GTCCT) was duplicated as previously described, indicating transposition at the target site. Furthermore, this ‘hot spot’ was also detected in isolates from Norway (2/8) and Denmark (17/20). The second insertion point detected in 45 isolates from the UK revealed integration into an Inc18-like plasmid, most likely by a process of target site recombination.

Conclusions: The presence of a common insertion point for isolates from different geographical areas could suggest the insertion of Tn1546 by transposition in a plasmid-specific site, followed by genetic rearrangement both inside the transposon and in the flanking regions.

Keywords: streptomycin adenyltransferase genes, Inc18 plasmids, transposons, E. faecium

Introduction

Enterococci are associated with the natural enteric flora of most mammals and birds. For decades, they have been used as indicators of faecal contamination of water and food for human consumption and are therefore of particular importance in food and public health microbiology.1 Enterococci show intrinsic resistance to cephalosporins, lincosamides and many β-lactams and low-level resistance to aminoglycosides. In addition, they have acquired resistance genes for many other classes of antimicrobials, such as tetracycline, chloramphenicol and glycopeptides, and have a high level of resistance to aminoglycosides.2

Enterococci were the first bacteria to be identified as having acquired glycopeptide resistance. A few years after glycopeptide-resistant clinical isolates of enterococci were reported in Europe,3 the use of the growth promoter avoparcin (a glycopeptide) in animal husbandry was also associated with occurrence of glycopeptide-resistant enterococci (GRE).4,5 High levels of GRE were found in production animals and in non-hospitalized humans.5–8 As a result, in 1997, a ban of this feed additive was enforced all across the EU. Conversely, in the USA, avoparcin has never been used, but a high frequency of GRE was detected in hospitals—most likely associated with the use of vancomycin to treat human infections.9

The most frequently found mechanism for glycopeptide resistance is the vanA operon, which is generally carried in transposon Tn1546, a non-conjugative transposon related to the Tn3 family.10 Tn1546 is transferred by replicative transposition, producing a 5 bp duplication at the target site.11 Transfer of resistance to susceptible enterococci is regularly mediated by integration in self-transferable plasmids, which can be disseminated by the conjugation process to other species.12,13
Tn1546 has been extensively characterized. Imperfect inverted repeats (36 of 38 bp) limit the 10.8 kb element that encodes the nine polypeptides responsible for the VanA resistance phenotype.\textsuperscript{14} Although \textit{vanA} genes are highly conserved, variations in parts of Tn1546 have been detected due to insertion sequences, deletions or point mutations. These variations have been used to subdivide the elements into distinct subgroups or types.\textsuperscript{15–17}

In this study, we have focused on the characterization of the integration site for Tn1546 in a panel of glycopeptide-resistant \textit{Enterococcus faecium} (GREF) recovered from broiler units across the UK and compared the results with a panel of isolates from Denmark, Norway and the Netherlands. The aim of this work was to identify specific ‘hot spots’ within a plasmid or chromosome where the insertion occurs and to clarify if these insertion points can be related to previously identified Tn types or to clonal lines that have been clustered using PFGE.

### Materials and methods

**Strain collection investigated**

A total of 150 GREF of broiler origin previously found to be carrying Tn1546-like elements was selected from the UK. They were collected from 19 unrelated farms representing 107 different PFGE types with diverse antibiograms. Of the 150 isolates, 26 were Tn type A, 57 were Tn type T, 38 were Tn type U, 1 was Tn type B, 4 were Tn type Y\textsuperscript{18} and 2 were Tn type T\textsuperscript{19} according to Palepou \textit{et al.}\textsuperscript{16} The remaining 24 isolates did not produce amplicons by the described long PCR (L-PCR). In addition, four isolates per year from 1995 to 1999 from the DANMAP collection (\textit{n} = 20) were included together with eight \textit{vanA}-GREF from Norway and five from the Netherlands.\textsuperscript{20} All isolates were of poultry origin.

**DNA digestion and ligation**

Two representatives were selected from the different Tn types (types T, U and A). Total DNA was digested with the \textit{Hin}I1 (AcyI) enzyme (Fermentas Life Science, Denmark), according to the manufacturer’s recommendations. \textit{Hin}I1 does not have a restriction target inside Tn1546. Circularization by ligation of the digested fragments was performed as described previously.\textsuperscript{16} Thereafter, 2 \(\mu\)L of the ligation mixture was used as a template for L-PCR with primers P745 (Table 1) and P1189 (5’-GAA CCW TGG CAT ATT CGC TAT G-3’), amplifying the flanking areas of Tn1546 (Figure 1). The PCR amplicons obtained were purified and sequenced, and two new primers were designed based on alignments of these new sequences.

**Polymerase chain reaction**

Two amplicons of different sizes were obtained. A set of primers (P1727 and P1728) was designed for sequences flanking Tn type A, and one primer (P1745) was designed for the downstream region of Tn type T. No sequence for the upstream region was obtained for Tn type T. Standard PCRs were run with the primer combinations described in Table 1 and Figure 1. The encoded sequences of the amplicons were identified by sequencing a representative number of isolates. Sequences were analysed using Vector NTI suite v8.0 and compared with those published in GenBank. Isolates that did not produce an amplicon were tested for variations at distal regions of Tn1546 (ORF1 and \textit{vanZ}) by PCR (data not shown).

### Results

Screening of isolates with primers, designed from the two different amplicons obtained from Tn type A and Tn-type T isolates, identified two main insertion points and a combination of both.

**First insertion point, streptomycin resistance gene (Figure 2)**

Upstream flanking Tn1546, primer combination P1727–P745. Seventy-seven of the UK isolates (\textit{n} = 150), 17 of the Danish isolates (\textit{n} = 20), 2 of the Norwegian isolates (\textit{n} = 8) and 1 Dutch isolate (\textit{n} = 5) produced an amplicon upstream of Tn1546 with this primer combination (Table 2). Thirty-six amplicons were sequenced and showed 100% homology with four streptomycin adenyltransferase genes, all harboured in different plasmids from Gram-positive bacteria: a plasmid sequence from \textit{Staphylococcus intermedius} (AY604739), \textit{Lactococcus lactis} sp. \textit{lactis} plasmid pK214 (X92946), \textit{Enterococcus casseliiflavus} plasmid TapA (AY939911) and \textit{Staphylococcus aureus} plasmid pS194 (X06627). In addition, these sequences also presented 100% homology with two partial sequences flanking the transposon in plasmids pVEF2 (AM410096) and pVEF1 (AM296544) isolated in Norway.\textsuperscript{21}

Downstream flanking Tn1546, primer combination P1728–P1189. Twenty-five of the UK isolates (\textit{n} = 150), 17 of the Danish isolates (\textit{n} = 20) and 2 of the Norwegian isolates (\textit{n} = 8)

### Table 1. Primer combinations designed to screen for insertion points to the left and the right of Tn1546

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>Sequence (5’ → 3’)</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upstream Tn</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1727</td>
<td>GTC TTT CTA TAT CCA TTC ATC TC</td>
<td>58</td>
<td>331</td>
<td>this work</td>
</tr>
<tr>
<td>P745</td>
<td>AAA GCT TAC CTA ACA CTA TAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Downstream Tn</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1728</td>
<td>ATT TGG AGA ATT GTT TAT ACA G</td>
<td>55</td>
<td>514</td>
<td>this work</td>
</tr>
<tr>
<td>P1715</td>
<td>GGA AAA CCG GTG ATA AAG</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P1745</td>
<td>CTT TTG ATT TGG TAC CTC TCA TC</td>
<td>58</td>
<td>484</td>
<td>this work</td>
</tr>
<tr>
<td>P1715</td>
<td></td>
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yielded amplicons with the primers used (Table 2). Sequenced amplicons from 36 of the isolates showed 100% similarity to the two partial sequences downstream of the transposon from plasmids pVEF2 and pVEF1. These sequences also exhibited 95% to 94% homology with the four streptomycin adenyltransferase genes detected upstream of Tn\textsubscript{1546} (\textit{S. intermedius} plasmid, pS194, pK214 and TnpA).

Furthermore, when adjoining the obtained sequences flanking Tn\textsubscript{1546} (158 bp to the left and 360 bp to the right), the resulting streptomycin adenyltransferase gene exhibited 96% homology to \textit{S. intermedius} plasmid AY604739. However, it also presented 95% similarity to the three streptomycin adenyltransferase genes described earlier (from pS194, pK214 and TnpA). The G + C content of the obtained sequence was 25%. 

Figure 1. Position of the primers inside of the transposon used in combination with the primers designed from the ligation products outside of the transposon (P1727, P1728, P1745).

**Figure 2.** Graphic representation of the different possibilities for the integration of Tn\textsubscript{1546} encountered in this study. \textit{n} is the number of isolates with the insertion point represented next to it. The total number of isolates tested was 183. X indicates that this insertion site was not found.
No sequence variations were found among the 36 sequenced amplicons examined, independently of country of origin or year of isolation. However, this partial sequence exhibited sequence variations in 20 nucleotides when compared with that published for S. intermedius (AY604739). Only five amino acid substitutions were detected in the protein sequence. The other 15 substitutions resulted in silent mutations and were all at the third base pair position of the codon (data not shown). The insertion of the transposon always occurred at the same spot, with the same orientation, and appeared to disrupt this unique streptomycin resistance gene. Where the insertion took place, transposition produced a 5 bp duplication of the target DNA (GTCCT), which is identical to the target sequence described previously.10

PCR screening using designed primers in 150 GREF UK isolates showed that 25 of the 26 A-types or wild-types had the same insertion point described earlier (Table 2). Furthermore, 19 of them were phenotypically resistant to streptomycin (MIC/C21 > 2048 mg/L), indicating the presence of a functional streptomycin resistance gene. Six isolates from a particular farm were susceptible (MIC, 128 mg/L). All 25 isolates were collected from eight unrelated broiler farms across the UK, and they presented 16 different PFGE profiles.

Second insertion point, Inc18-like plasmid (Figure 2)

Upstream flanking Tn1546. Sequence polymorphisms inside the flanking region left of Tn1546 prevented the L-PCR from producing an amplicon from the ligation products. Despite several approaches being tried, the sequence upstream of Tn1546 (data not shown), no readable results could be obtained.

Downstream flanking Tn1546, primer combination P1715–P1745. Based on sequenced amplicons, a second successful primer set was designed for downstream of the transposon. Twelve amplicons were sequenced for further investigation. The flanking downstream region for the integration point of Tn1546 showed high similarity with sequences from the Inc18 plasmid family. Sequenced amplicons exhibited 100% homology to the pAM-b1 plasmid (AF007787) just upstream of the copF gene (a copy number repressor). Moreover, these sequences also showed 99% identity with the pRE25 plasmid (X92945), 97% with pIP501 (AJ505823), which are associated with Enterococcus faecalis and 93% with pMD101 (X66468) and pSM19035 (AY357120) associated with Streptococcus pyogenes. At position 34334 of the pRE25 plasmid, just 27 bp upstream of the junction of the presumed integration point of Tn1546, an A-T rich segment was found, which shared similarities with the inverted repeats of Tn1546. In particular, a 6 bp nucleotide sequence (GTAAAT) was detected, showing 100% homology between the pRE25 segment and the transposon (Figure 3). Duplication of the target site was not observed for this insertion junction.

A direct repeat of 21 bp (AAA TCA CAA GTG ATT AAT) was identified for five isolates in consensus with the pRE25 sequence, whereas the other seven only contained one copy. This small DNA fragment seems to be repeatedly associated with a putative omega protein-binding region or a copy number repressor in plasmids from different species (E. faecium pVEF2 plasmid, Pediococcus acidilactici plasmid pEOC01, E. faecalis plasmid pSL1, S. pyogenes plasmid pSM19035, Streptococcus agalactiae
plasmid pIP501 and Clostridium difficile). No other differences were observed among sequences on that side of Tn1546.

Of the 150 isolates from the UK, 105 produced an amplicon with this primer combination (Table 2). They were all recovered from 19 unrelated broiler farms, and they presented 50 different PFGE profiles. This insertion site appeared to be common for Tn types U and T. None of the isolates from Denmark, Norway or the Netherlands tested positive for this primer combination.

Third insertion point, streptomycin resistance gene upstream and Inc18-like plasmid downstream (Figure 2)

Primer combination P1727–P745 upstream and P1715–P1745 downstream. Finally, 45 isolates tested positive using PCR for the combination of the two insertion points already described: streptomycin adenyltransferase gene upstream of Tn1546 and Inc18-like plasmids downstream (Table 2). They were recovered from 15 different farms across the UK and exhibited 30 different PFGE types.

In addition, seven isolates from the UK and one from the Netherlands were identified, which had the streptomycin adenyltransferase gene upstream of the integration point of Tn1546, but the insertion downstream could not be assigned. Nevertheless, 13 isolates from the UK did not produce an amplicon on either the left or the right side of the transposon. All but one were non-typeable by L-PCR. These 13 isolates were tested for internal variations at the terminal regions of Tn1546; 6 of them presented deletions at both ends of the transposon, and the other 7 had a conserved vanZ and variations in the ORF1 region (data not shown).

Discussion

This study was performed to determine the insertion point(s) of the vanA-encoding Tn1546. Within a very polymorphic panel of GREF isolates of poultry origin, two common insertion points were detected. The insertion point inside a streptomycin resistance gene appeared to be distinctive of the wild-type transposon among the tested isolates, representing several clones based on previous PFGE types. Furthermore, the same insertion point was detected among isolates from Denmark and Norway, collected from 1995 to 1999. When aligning sequenced amplicons, target duplication was detected, indicating transposition as the mechanism for integration. However, this ‘hot spot’ was not detected in any other tested Tn types (T and U) that have presumably undergone an evolution by deletion and point mutations from the wild-type, even though some of them did reveal sequences identical to the streptomycin resistance gene downstream of the integration point. No target duplication was found at these integration points, which could indicate a secondary event not resulting from transposition. In addition, this second insertion point into an Inc18-like plasmid was only detected in isolates from the UK, which could indicate the presence of certain selective pressure acting in these farms.

No other primers for the upstream region could be designed to be combined with the downstream integration sequence in pRE25, since sequencing of obtained amplicons yielded unreadable results. Further analysis of a number of isolates, with primers targeting internal regions of Tn1546, revealed that internal variations in the transposon were quite common, especially for Tn types U and T. These variations might explain the lack of success in finding an upstream flanking sequence. On the other hand, there is also the possibility of a non-specific insertion site.

However, as described previously for mechanisms of transposition for conjugative transposons,22 integration of Tn1546 into a plasmid such as that resembling a plasmid of the Inc18 family (pAM-β1, pRE25 and pIP501) could be a possible explanation for the third insertion point, where some isolates presented the streptomycin resistance gene flanking upstream of the Tn1546 and pRE25 flanking downstream. We propose that the A-T rich fragment and the short regions, which exhibited similarities between the ends of the transposon and the pRE25 by the ORF39, could have led to insertion of the transposon by a process of target site recombination. Similar target specificity regions have been described previously for conjugative transposons.23 During excision, the transposon could have mobilized the left end of the streptomycin resistance gene at the original insertion point. Taking into consideration that the wild-type transposon (Tn type A) in most cases (25/26) was inserted in the same location, we propose that Tn types T and U could have evolved from this prototype subsequently; since plasmid rearrangements through transpositions and homologous recombination are frequent, it makes them highly dynamic structures.24 Nevertheless, further work is required to understand accurately the whole process of excision, translocation and insertion into the Inc18-like plasmids if this is the case.

Put together, these results may suggest dissemination of the same mobile genetic elements across different clonal lines not only within the same farm environment but also within the broiler sector. A common supplier of birds to the different countries in Europe or a common stock provider could be a likely explanation for the spread of these seemingly related mobile genetic elements resulting from a few evolutionary genetic events. Still, larger epidemiological studies involving breeding companies, hatcheries, and feed and litter producers combined with the characterization of genetic mobile elements

Figure 3. Insertion junction downstream of Tn1546. See the regions of homology between the end of the transposon and the fragment of the plasmids. The plasmid pRE25 has a 6 bp homology followed by small regions of similarities with the transposon (fragments inside the boxes).
from a significant and comparable panel of GREF strains recovered from different countries would clarify the reality of this hypothesis.

The origin of these mobile genetic elements and the origin of the first presumed transposition event remain unknown. The G + C content (~25%) of the presumed plasmid-borne streptomycin adenyltransferase gene into which the transposon was inserted exhibited a much lower G + C content than the one for E. faecium (38%) or Tn1546 (44%), indicating a non-enterococcal origin. It is known that the exchange of mobile genetic elements between different species plays an important role in bacterial evolution.25,26 This is exemplified by the high-sequence homology exhibited by fragments harboured in plasmids from different species. The mutations observed in the third nucleotide of the streptomycin resistance gene, when compared with the published sequences, could illustrate a mechanism induced by enterococci for the stabilization of acquired exogenous DNA. None of these mutations increased the G + C content as observed in other species.27

The origin of the glycopeptide resistance genes still remains unclear.28,29 Screening for insertion points in glycopeptide-producing organisms and in non-glycopeptide-producing organisms that have developed similar mechanisms to defend themselves may provide a successful tool to clarify the origin and evolution of these resistance genes. Moreover, since gene transfer among Gram-positive cocci is a frequent event,52 the presence of an insertion point could be crucial for the identification of potential recipients of specific genetic mobile elements, especially if these insertion sites are also present in more pathogenic bacteria. Therefore, ‘hot spots’ could be used as fingerprints in the molecular examination of vanA gene cluster evolution and transmission.

To conclude, two specific insertion points for Tn1546 were found among isolates from unrelated farms across the UK. Furthermore, the insertion in the presumptive streptomycin adenyltransferase gene was detected in isolates from Norway and Denmark and exhibited a 5 bp direct repeat as previously described in a human isolate.10 The diversity of clonal lines compared with the diversity of transposon types and insertion points also suggests horizontal transmission of seemingly related mobile genetic elements. There are still gaps in our knowledge that need to be addressed, emphasizing the need for further studies on the genetic background of the resistances, mobile genetic elements involved in the transmission of these resistance genes, origin and evolution as well as the selective environmental pressures that have led to the persistence and spread of these elements over recent years.

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Transparency declarations

None to declare.

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