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# Identification of Tn*5397*-like and Tn*916*-like transposons and diversity of the tetracycline resistance gene *tet*(M) in enterococci from humans, pigs and poultry

**Yvonne Agersø[1]*, Anders Gorm Pedersen[2] and Frank Møller Aarestrup[1]**

[1]*Danish Institute for Food and Veterinary Research, 1790 Copenhagen V, Denmark;*
[2]*Center for Biological Sequence Analysis, The Technical University of Denmark, 2800 Lyngby, Denmark*

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***Objectives*: To analyse the sequence diversity of the tetracycline resistance gene *tet*(M) and its location on mobile elements in *Enterococcus faecium* and *Enterococcus faecalis* from humans, pigs and poultry in Denmark.**

***Methods*: A total of 76 isolates were screened for Tn*916*/Tn*1545*-like and Tn*5397*-like transposons using PCR. *tet*(M) was sequenced in 15 of the isolates and compared with *tet*(M) sequences submitted to GenBank (phylogenetic analysis and signs of recombination). Plasmids were extracted, filter-mating experiments were performed and Tn*5397*-like transposons were further characterized in selected isolates.**

***Results*: In 8 of 13 isolates of *E. faecium* from broilers, *tet*(M) was present on Tn*5397*-like transposons, whereas *tet*(M) was predominantly associated with Tn*916*/Tn*1545*-like transposons in *E. faecium* from pigs and humans, as well as in *E. faecalis* from humans, pigs and broilers (50 of 63 isolates). The *tet*(M) genes were divided into three major subgroups according to the phylogenetic analysis. Subgroup I consisted of *tet*(M) from *Clostridium difficile* and *E. faecium* associated with Tn*5397*-like elements, subgroup II consisted of *tet*(M) located on Tn*916*/Tn*1545* family transposons and subgroup III consisted of *tet*(M) associated with composite elements containing several resistance genes. We found evidence of recombination both within and between these groups. Moreover, we identified an *E. faecium* isolate with both Tn*916*/Tn*1545*-like and Tn*5397*-like elements.**

***Conclusions*: This study showed that enterococci contain diverse *tet*(M) genes present on different mobile elements, which may suggest that enterococci play an important role in the evolution and horizontal spread of mobile elements carrying *tet*(M). This is the first report of Tn*5397*-like elements in enterococci.**

Keywords: *Enterococcus*, *tetA*(M) diversity, Tn*5397*

## Introduction

Enterococci isolated from animals, humans and other sources are often resistant to tetracyclines, and several classes of tetracycline resistance genes have been identified in enterococci. In a previous study, the *tet*(M) gene was found in 95% of *Enterococcus faecium* and *Enterococcus faecalis* from humans, pigs and broilers.[1] This gene is widely distributed and has to date been found in 42 genera of Gram-positive and Gram-negative species.[2] This is probably due to the association of the *tet*(M) gene with conjugative elements.[2,3] In enterococci and other species, *tet*(M) has been found to be associated with conjugative transposons related to the Tn*916*/Tn*1545* family.[3] Other conjugative transposons such as Tn*5397* from *Clostridium difficile* have been found to harbour *tet*(M) as well.[4] Tn*5397* is a 21 kb tetracycline resistance-encoding conjugative transposon found originally in *C. difficile*.[4,5] Tn*5397* was shown to be transferred by a conjugation-like process from *C. difficile* to *Bacillus subtilis* and back to *C. difficile* and between *C. difficile* strains.[5] Tn*5397* is related to Tn*916*; the central regions that are involved in conjugation of these two elements are very similar.[4,5] However, Tn*5397* can be distinguished from Tn*916* by at least two important characteristics: first, Tn*5397* contains a group II intron inserted into a gene almost identical to *orf14* from Tn*916*, and, second, the DNA sequences at the ends of Tn*5397* are completely different from those of Tn*916*.[4] Instead of possessing the *int* and *xis* genes that have been shown to be required for integration and excision of Tn*916*, Tn*5397* contains the gene *tndX*,

*Corresponding author. Tel: +45-72-34-60-00; Fax: +45-72-34-60-01; E-mail: ya@dfvf.dk



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which encodes a putative protein not related to Int or Xis but belonging to the large resolvase subgroup of site-specific recombinases.[4,6]

Although, *tet*(M) is widely distributed among bacteria in different environments, only a few studies concerning the diversity and phylogenetic relationship of the genes have been performed. In one analysis, based on high-resolution restriction analysis of the *tet*(M) gene in tetracycline-resistant clonal lineages of *Streptococcus pneumoniae*, six allele types were identified, and these were all in isolates containing the *int*-Tn gene of the Tn*916*/Tn*1545* conjugative transposons.[7]

In another study, restriction enzyme analysis and partial sequencing of the *tet*(M) gene in *Lactobacillus* isolated from different types of fermented dry sausage revealed two different allele types.[8] The *tet*(M) genes were mainly located on plasmids.[8] Recently, Huys *et al.*[9] characterized enterococci isolated from food. All isolates that contained *tet*(M) had the *xis*-Tn gene from Tn*916*/Tn*1545* family transposons. The *tet*(M) genes were classified into four distinct groups.[9]

The present study was conducted to characterize and determine the occurrence of mobile elements associated with *tet*(M) in *E. faecium* and *E. faecalis* isolated from humans, pigs and broilers in Denmark, and to see whether there is a correlation between the presence of certain mobile elements and the diversity of *tet*(M) genes at the nucleotide level by comparison with *tet*(M) sequences submitted to the GenBank database.

## Materials and methods

### *Bacterial strains*

A total of 76 bacterial isolates from broilers, pigs and humans were obtained from the continuous surveillance programme for antimicrobial resistance in Denmark (DANMAP), as described previously.[10] Of the 76 isolates, 40 were *E. faecalis* isolates (13 from humans, 15 from pigs and 12 from broilers) and 36 were *E. faecium* isolates (10 from humans, 13 from pigs and 13 from broilers). The 17 enterococcal isolates (Table 1) included in the phylogenetic analysis had very distinct PFGE patterns, indicating the absence of any clonal relationship between these strains.

All isolates were tested for susceptibility to bacitracin, chloramphenicol, erythromycin, gentamicin, kanamycin, penicillin, streptomycin, quinupristin/dalfopristin, tetracycline and vancomycin and for the presence of selected resistance genes, including *tet*(M), in a previous study.[1] The isolates from pigs and broilers were collected during the first 9 months of 1998. Only one isolate per flock or herd was included in the collection.[1] Bacterial isolates from humans were obtained from stool samples received at Statens Serum Institut, Denmark, for diagnostic purposes, as described previously.[1]

### *Detection of Tn916-like and Tn5397-like conjugative transposons*

PCR was used to demonstrate the presence of the Tn*916*-like or Tn*5397*-like transposons in the 76 isolates containing *tet*(M). For the detection of Tn*916*-like transposons, the *xis*-Tn gene of Tn*916*

**Table 1.** Bacterial strains and isolates used in this study

| Isolate/GenBank accession no. | Source | Other resistance phenotypes | Mobile element carrying *tet*(M) | Other resistance genes | Reference |
|---|---|---|---|---|---|
| *Enterococcus faecalis* | | | | | |
| 9830457-1/DQ223239 | broiler | | Tn*916*/Tn*1545*-like | | this study |
| 9830479-1/DQ223240 | broiler | | Tn*916*/Tn*1545*-like | | this study |
| 9830090-3/DQ223249 | pig | $Erm^R$, $Str^R$ | Tn*916*/Tn*1545*-like | *erm*(B) | this study |
| 9830089-2 | pig | $Erm^R$, $Str^R$ | | *erm*(B) | this study |
| 98300091-1 | pig | $Erm^R$ | | *erm*(B) | this study |
| 20074-s-1/DQ223248 | human | $Erm^R$, $Str^R$, $Km^R$, $Chl^R$ | Tn*916*/Tn*1545*-like | *erm*(B), *aphA-3* | this study |
| 20028-s-1/DQ223247 | human | $Erm^R$, $Str^R$, $Km^R$ | Tn*916*/Tn*1545*-like | *erm*(B), *aphA-3* | this study |
| 18854-s-1/DQ223241 | human | | Tn*916*/Tn*1545*-like | | this study |
| *Enterococcus faecium* | | | | | |
| 9830414-1/DQ206711 | broiler | $Erm^R$ | Tn*5397*-like | *erm*(B) | this study |
| 9830409-1/DQ223244 | broiler | $Erm^R$ | 80 kb plasmid | *erm*(B) | this study |
| 9830359-1/DQ223250 | broiler | $Erm^R$ | Tn*5397*-like | | this study |
| 9830133-1 | broiler | $Bac^R$, $Erm^R$, $Str^R$, $Km^R$, $Pen^R$ | Tn*916*/Tn*1545*-like and Tn*5397*-like | ND | this study |
| 9830491-2/DQ223245 | pig | $Erm^R$ | Tn*916*/Tn*1545*-like | *erm*(B) | this study |
| 9830498-4/DQ223242 | pig | $Erm^R$ | Tn*916*/Tn*1545*-like | *erm*(B) | this study |
| 9830470-4/DQ223243 | pig | $Erm^R$ | 80 kb plasmid | | this study |
| 18836-s-2/DQ223238 | human | $Erm^R$, $Str^R$, $Km^R$, $Pen^R$ | Tn*916*/Tn*1545*-like | *erm*(B), *aphA-3* | this study |
| 15109-s-2/DQ223251 | human | $Erm^R$ | Tn*916*/Tn*1545*-like | *erm*(B) | this study |
| 20032-s-1/DQ223246 | human | $Erm^R$, $Str^R$, $Km^R$, $Chl^R$ | Tn*916*/Tn*1545*-like | | this study |
| Controls | | | | | |
| *Bacillus subtilis* CU2189 | | $Tet^R$ | Tn*5397* | | 4 |
| *E. faecalis CG110* | | $Tet^R$ | Tn*916* | | 11 |

$Bac^R$, bacitracin resistant; $Chl^R$, chloramphenicol resistant; $Erm^R$, erythromycin resistant; $Km^R$, kanamycin resistant; $Pen^R$, penicillin resistant; $Str^R$, streptomycin resistant; $Tet^R$, tetracycline resistant; ND, not determined.

was amplified using the primers Tn916-1 and Tn916-2 (PCR conditions: 3 min hot start at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 45°C, 1 min at 72°C and a final extension for 10 min at 72°C). Eleven of the *xis*-Tn gene positive isolates were checked by linking the essential *xis*-Tn gene of Tn*916* to the *tet*(M) gene using primers Tn916-2 and ReversTetM-2, as described previously (PCR conditions: 3 min hot start at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 45°C, 3 min at 72°C and a final extension for 10 min at 72°C).[12]

For the detection of Tn*5397*-like transposons, the *tndX* gene of Tn*5397* was amplified using PCR with primers Tn5397-tndx-1 and Tn5397-tndx-2 (PCR conditions: 3 min hot start at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 53°C, 3 min at 72°C and a final extension for 10 min at 72°C). DNA sequencing was used to verify the identity of the gene products in two randomly selected isolates (9830414-1 and 9830359-1).[13] In all *tndX*-positive isolates *tndX* was linked to the *tet*(M) gene using four PCR sets with the following primers: (i) ReversTetM-2 + Tn5397; (ii) Tn5397-6 + Tn5397-7; (iii) Tn5397-4 + Tn5397-5; and (iv) Tn5397-tndX-1 + Tn5397-tndX-2. The PCR products of 9830414-1 were sequenced, assembled and submitted to GenBank (accession no. DQ206711). The ability of Tn*5397* and Tn*916* to excise from the genome to form a circular form was demonstrated using the following primer sets: REO + LEO and 2tn5397 + 3tn5397, amplifying the circular form of Tn*916*-like and Tn*5397*-like transposons, respectively.

A reference strain containing the respective transposon was included as a positive control for each PCR assay (Table 1). For negative controls both samples containing the PCR mixture with no DNA and DNA from the reference strain containing another conjugative transposon were used. The primers used are listed in Table 2.

### *Filter-mating experiments*

Filter-mating experiments were performed as described previously using four donors (*E. faecalis* 9839133-1 and *E. faecium* 9830470-4, 9830409-1 and 9830414-1) to the recipients *E. faecium* BM4105 and *E. faecalis* JH2-2.[14] Transconjugants were selected on brain heart infusion agar supplemented with tetracycline 8 mg/L, rifampicin 25 mg/L (Sigma-Aldrich, Brøndby, Denmark) and fusidic acid 25 mg/L (Sigma-Aldrich). *tet*(M), Tn*5397* and Tn*916* were verified in the transconjugants as described above and the PFGE patterns of selected transconjugants were compared with the PFGE patterns of the respective recipient and donor.

### *PCR amplification of full-length tet(M)*

The upstream part of the gene was amplified using PCR in one fragment (primers: TetM-upstream and TetM-up, PCR product 1, Table 2), and in isolates where *tet*(M) was located on Tn*916*-like transposons overlapping with the upstream PCR fragment, the downstream part of the gene was amplified also in one fragment (primers: ReversTetM-2; Tn916-2, PCR product 2a, Table 2) (Figure 1).[12]

In one isolate where the downstream part of *tet*(M) could not be determined, the DNA was digested using restriction enzyme *Hin*dIII and ligated. The circularized fragment was used as the template for PCR with primers pointing out of the gene (primers: ReversTetM-1; ReversTetM-2, Table 2) in order to determine the sequence downstream of *tet*(M). The DNA sequence downstream of *tet*(M) revealed using this method showed 99.6% homology to the corresponding sequence of transposon Tn*5397* from *C. difficile*, position 16 571–17 045 (GenBank accession no. AF333235). The sequence was used to design a new primer (Tn5397) and the downstream fragment PCR 2b was amplified from isolates with this sequence (Figure 1).

**Table 2.** Primers used for the detection of *xis*-Tn gene encoding exitase from Tn*1545*/Tn*916*-like transposons; *tndX* gene encoding resolvase from Tn*5397* transposons; amplification and sequencing of full-length *tet*(M) gene

| Primer | Sequence (5′–3′) | Reference |
|---|---|---|
| TetM-upstream | 5′-TTGAATGGAGGAAA-ATCAC-3′ | this study |
| Tet(M)-1 | 5′-GTTAAATAGTGTT-CTTGGAG-3′ | 1 |
| TetM sequence-1 | 5′-TACTTTCCCTAAGA-AAGAAAGT-3′ | this study |
| TetM sequence-2 | 5′-TGGAGCGATTACAG-AATTA-3′ | this study |
| TetM sequence-3 | 5′-GCAGAAATCAGTAG-AATTGC-3′ | this study |
| TetM sequence-4 | 5′-TTAACTCTGTTAAAC-AAACACTA-3′ | this study |
| TetM sequence-5 | 5′-TCACCATTTATTGAA-GTATACAT-3′ | this study |
| TetM-up | 5′-CTGGCAAACAGGTTC-3′ | this study |
| ReversTetM-1 | 5′-CTCCAAGAACACTATTTAAC-3′ | this study |
| ReversTetM-2 | 5′-TTGTTAGAGCCATAT-CTTAG-3′ | 12 |
| Tn916-2 | 5′-CTAGATTGCGTCCAA-3′ | 12 |
| Tn916-1 | 5′-GCCATGACCTATCTT-ATA-3′ | this study |
| Tn5397-tndX-1 | 5′-ATGATGGGTTGGACA-AAGA-3′ | this study |
| Tn5397-tndX-2 | 5′-CTTTGCTCGATAGGC-TCTA-3′ | this study |
| Tn5397 | 5′-CATTTAATTCAAATC-TGTATTAA-3′ | this study |
| Tn5357-4 | 5′-CCGCTGAATCCATCA-TCAATG-3′ | this study |
| Tn5357-5 | 5′-TTGGAAATGCTATTC-GTGCTGTA-3′ | this study |
| Tn5397-6 | 5′-TTTGTCTATTGCTGG-TGGATAACT-3′ | this study |
| Tn5397-7 | 5′-GGCTTCTTGTTAAAC-GAAACG-3′ | this study |
| LEO | 5′-GGTTTTGACCTTGAT-AAAGTGTGATAAGTCC-3′ | 6 |
| REO | 5′-CGAAAGCACATAGAA-TAAGGCTTTACGAGC-3′ | 6 |
| 2tn5397 | 5′-ACAACCAGCAGGA-AAACAGG-3′ | 6 |
| 3tn5397 | 5′-ACGTGTATCAAGCA-GAGGGAATCGGTAAA-3′ | 6 |

### *Sequencing of the tet(M) gene*

DNA sequencing of PCR products was preformed on an ABI 377A automatic sequencer using the PRISM BigDye terminator kit (Applied Biosystems, Foster City, CA, USA) with the primers listed in Table 2, as described previously.[1,13] The *tet*(M) gene in isolates containing Tn*1545*/Tn*916*-like or Tn*5397*-like elements was sequenced as illustrated in Figure 1 using the primers listed in Table 2. All sequences submitted to GenBank were sequenced twice.

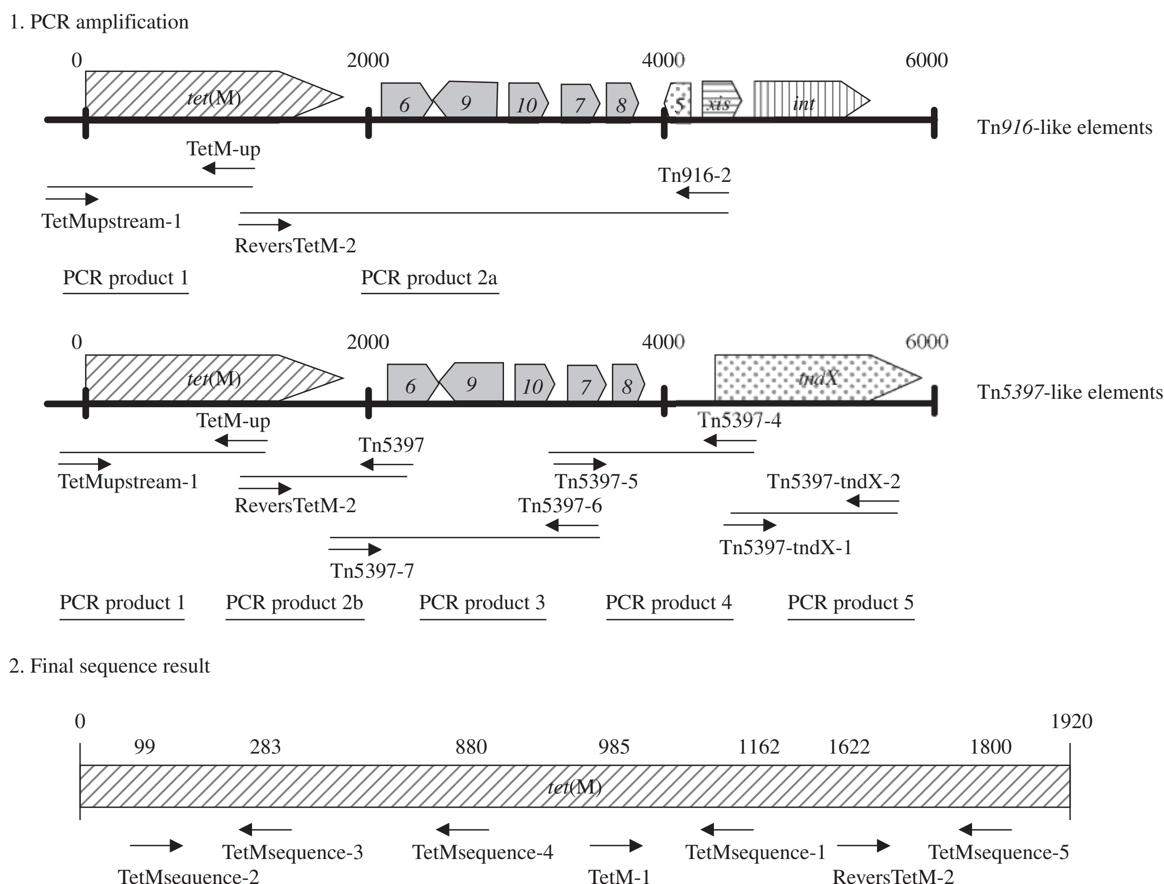


**Figure 1.** Strategy for sequencing *tet*(M) genes in Tn*1545*/Tn*916*-like and Tn*5397*-like transposons. The arrows indicate the direction of the primers used.

### *Construction of phylogenetic trees*

The 15 sequences obtained in this study were compared with full-length and partial *tet*(M) DNA sequences retrieved from GenBank using the Blast program 2.0. All partial sequences covering positions 286 to 1543 from the translation start site were included. DNA sequences from uncultured samples or sequences with no description of the organism or origin of the DNA were not included.

Phylogenetic trees were reconstructed using Bayesian techniques as implemented in the program MrBayes, version 3.0B4.[15] The program MrModeltest was used to find the most appropriate model for the process.[16] In both the reconstructed trees [full-length and partial *tet*(M) sequences, respectively] the best model was found to be HKY + I + G, the so-called HKY model, with a proportion of invariable sites and gamma-distributed rate variation across sites.[17] MCMC sampling was performed for 5 000 000 generations with four chains.

Convergence was confirmed by comparing the results of two independent runs. The program Tracer was used to determine burn-in and for further confirmation of proper mixing and adequate run-length.[18]

### *Detection of signs for recombination within the tet(M) group*

To search for signs of recombination we used several independent tools. The maxchi2 program implements a modification of the maximum chi-square method.[19] When assessing whether sequence mosaics were more pronounced than expected for random reasons, 1000 alignment permutations were used. We also used the program TOPALi, which implements a number of different methods.[20] Specifically, we used the probabilistic divergence measure method of Husmeier and Wright[21] and the Hidden Markov model method of Husmeier and McGuire.[22] In addition to these specialized methods, we also searched for local sequence similarities using the BLAST local alignment and database search program.[23]

## Results

### *Detection of Tn916-like and Tn5397-like conjugative transposons*

A total of 39 *tet*(M)-positive *E. faecium* and 37 *E. faecalis* isolated from humans, pigs and broilers were screened for the presence of the *xis*-Tn gene from Tn*916*-like transposons and the resolvase gene *tndX* from the transposon Tn*5397* (Table 3). One of the two conjugative transposons could be detected in 62 (82%) of the isolates. The *xis*-Tn gene from Tn*916*-like transposons was present in both *E. faecium* and *E. faecalis* from all three sources (humans, pigs and broilers). Of the isolates from humans, 21 (91%) contained the *xis*-Tn gene from Tn*916*-like transposons. *E. faecium* from broilers contained the lowest fraction of the *xis*-Tn gene from Tn*916*-like transposons, present in four isolates (31%), and the resolvase gene *tndX* from the transposon Tn*5397* was present in eight (62%) of the isolates. The PCR products obtained from two isolates (9830414-1 and 9830359-1) were sequenced and revealed 100% identity to the corresponding

**Table 3.** Distribution of Tn*1545*/Tn*916*-like and Tn*5397*-like transposons among enterococci isolated from broilers, pigs and humans in Denmark

| | *xis*-Tn gene from Tn*1545*/Tn*916* | *tndX* gene from Tn*5397* | No transposons detected |
|---|---|---|---|
| *E. faecalis* | | | |
| humans | 12/13 (92%) | 0/13 | 1/13 (8%) |
| pigs | 8/15 (53%) | 0/15 | 7/15 (47%) |
| broilers | 10/12 (83%) | 0/12 | 2/12 (17%) |
| *E. faecium* | | | |
| humans | 9/10 (90%) | 0/10 | 1/10 (10%) |
| pigs | 11/13 (85%) | 0/13 | 2/13 (15%) |
| broilers | 4/13 (31%) | 8/13 (62%) | 1/13 (8%) |
| Total | 54/76 (71%) | 8/76 (11%) | 14/76 (18%) |

sequence in *C. difficile* (GenBank accession no. AF333235, position 19115–19704). One of the *E. faecium* isolates (9830133-1) from broilers contained both the *xis*-Tn gene from Tn*916*-like transposons and *tndX* from transposon Tn*5397*. Both genes could be linked to *tet*(M). The highest fraction of isolates [seven isolates (47%)] with no detectable mobile elements was found among *E. faecalis* from pigs.

### *Characterization of Tn5397-like transposons*

The eight isolates containing *tndX* were further characterized. *tet*(M) could be linked to *tndX* and gave the expected size of product for all PCR set-ups except in two isolates (9830133-1 and 9830359-1) where the PCR product (tn5397-6; tn5397-7) gave a band ~1500 bp larger than expected. In seven isolates the circular form of Tn*5397* was detected. No PCR product of the circular form of Tn*5397* was detected in the isolate with both transposons (9830133-1), but the circular form of Tn*916* was detected. Isolate 9830133-1 was used as a donor in filter-mating experiments, and horizontal gene transfer of *tet*(M) was demonstrated to both recipients (*E. faecium* BM4105 $2.1 \times 10^{-8}$ transconjugant/donor and *E. faecalis* $10^{-8}$ transconjugant/donor), but all transconjugants contained only Tn*916*.

The *E. faecium* isolate (9830414-1) from broilers with a Tn*5397*-like element was partially sequenced. The sequence including *tet*(M) and *tndX* genes (5070 bp) had 97% identity to the corresponding sequence of *C. difficile* (GenBank accession no. AF333235, position 14631–19700). Isolate 9830414-1 was used as a donor in filter-mating experiments and Tn*5397* was transferred to *E. faecium* BM4105 ($7 \times 10^{-8}$ transconjugant/donor) but not to *E. faecalis* JH2-2 (detection limit $>1.1 \times 10^{-8}$ transconjugant/donor).

### *Sequencing of the tet(M) gene*

In order to sequence *tet*(M) in isolates with Tn*916*-like transposons an upstream and a downstream PCR product of the gene were amplified according to the sequencing strategy illustrated in Figure 1. Eleven of the isolates could be sequenced using this strategy. In six isolates fragment 2a could not be amplified using this approach. PCR for circularized DNA from one of these isolates revealed *tet*(M) on a Tn*5397*-like element and the downstream part of the gene was sequenced. One more isolate (9830359-1) had *tet*(M) on a Tn*5397*-like element, and a downstream PCR fragment (2b, Figure 1) was amplified and sequenced from this isolate. Circularized DNA from two isolates revealed the downstream sequence of *tet*(M) but no transposon was detected in these isolates. Two isolates failed to be amplified and full-length *tet*(M) could not be sequenced. These were *E. faecalis* isolates from pigs.

### *Construction of phylogenetic trees*

Full-length *tet*(M) sequences were obtained from 15 isolates and the sequences were aligned with 25 full-length *tet*(M) sequences retrieved from GenBank. This alignment was then used to construct a phylogenetic tree (Figure 2). In the resulting tree, *tet*(M) fell into three distinct subgroups. Subgroup I of *tet*(M) had a clade-credibility of 100% and contained *tet*(M) on Tn*5397* from *C. difficile* (AF333235), two *E. faecium* isolates from broilers with Tn*5397*-like elements and *tet*(M) from *E. faecium* isolated from pig and broiler with *tet*(M) associated with 80 kb plasmids (data not shown). Subgroup II had a clade-credibility of 99% and contained 11 isolates with *tet*(M) on Tn*916*-like elements, where 8 were 100% identical to *tet*(M) on Tn*916* from *E. faecalis* (GenBank accession nos. X92947 and M85225). These isolates were from *E. faecium* isolated from pigs and humans and from *E. faecalis* isolated from broilers and pigs. Two *tet*(M) genes of *E. faecalis* from humans were 100% identical to each other and branched separately from the other *tet*(M) genes associated with Tn*916*-like elements. The clade-credibility of subgroup III was 54%, indicating only limited support for its monophylicity. Included in the group is one *tet*(M) gene derived from an *E. faecalis* isolate of human origin (20074-s-1). This *tet*(M) gene is most similar to *tet*(M) from Tn*1545* (GenBank accession no. X04388). The isolate was also resistant to erythromycin, streptomycin and kanamycin and contained the *erm*(B) and *aphA3* genes. The resistance patterns and additional resistance genes for the isolates are listed in Table 1.

A second phylogenetic tree was constructed from an alignment of 63 partial *tet*(M) sequences in GenBank and the corresponding segment of our 15 *tet*(M) sequences (position 286–1443 from the translation start was used). This included *tet*(M) of *Lactobacillus* from fermented sausages and enterococci primarily from cheese. The phylogenetic tree had the same structure as the full-length tree, with three major groups (data not shown). Again there was good support for the monophylicity of both group I and group II (100 and 93%, respectively), while the monophylicity of group III received only limited support (57%). The additional sequences aligned with subgroup II, except the *tet*(M) genes of two aquatic isolates from *Vibrio* sp. (GenBank accession no. AB124557) and *Photobacterium damselae* (GenBank accession no. AB124556), respectively, which were in subgroup III. *tet*(M) of two *E. faecalis* isolated from cheese (GenBank accession nos. AJ585083 and AJ585082) branched together with those of two *E. faecalis* isolated from humans (18854-s-1 and 20028-s-1) within subgroup II. All four isolates contained the *xis*-Tn gene of the Tn*916*/Tn*1545* family.

### *Detection of signs for recombination within the tet(M) group*

Using the maxchi2 program on the entire full-length alignment very strongly indicated that recombination was indeed present.

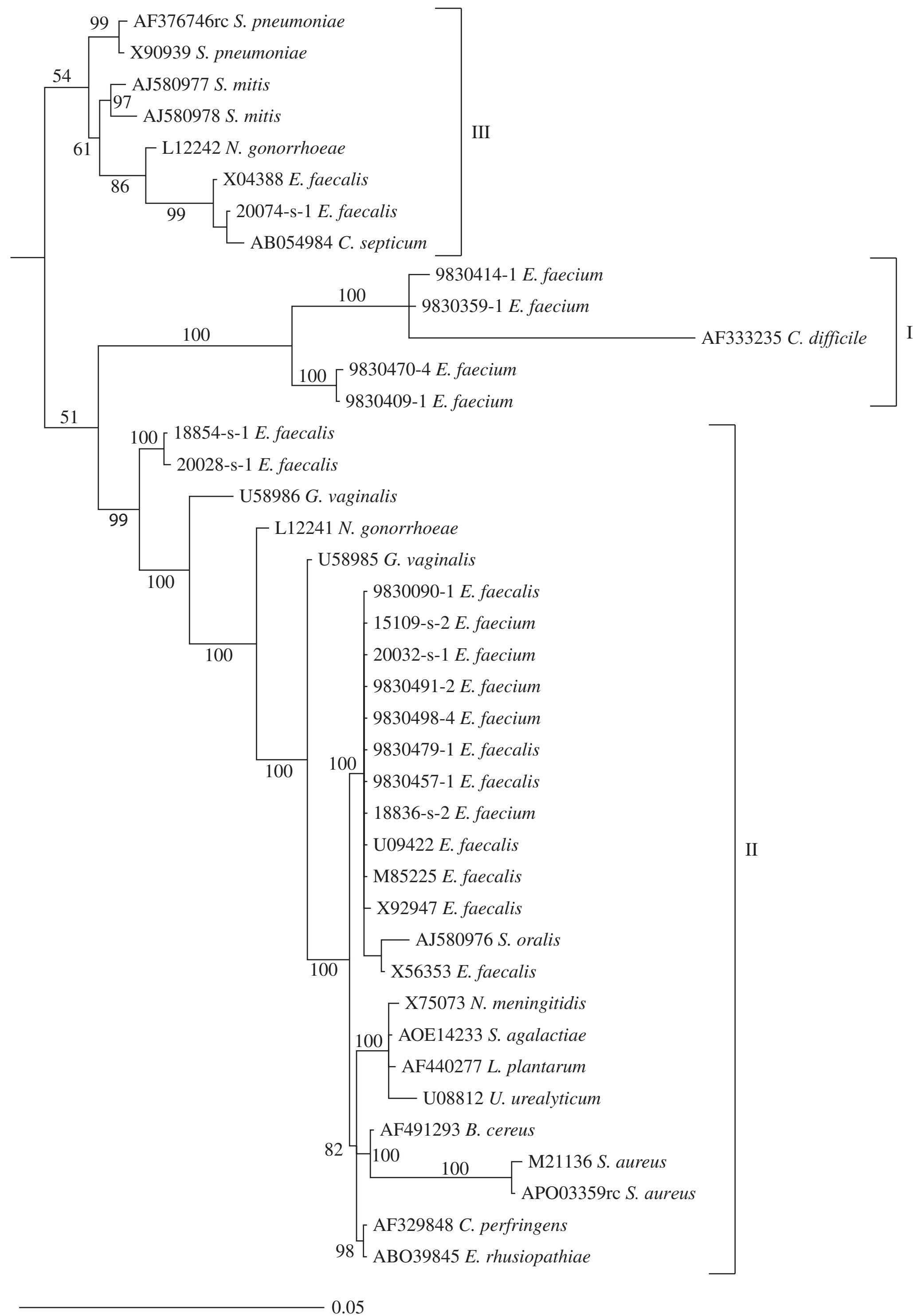


**Figure 2.** Phylogenetic tree of full-length *tet*(M) sequences in comparison with *tet*(M) sequences deposited in the GenBank/EMBL/DDBJ database. Statistically significant support (clade-credibility) for a branch is indicated at the node as a percentage. The branch lengths are scaled in proportion to the extent of the change per position as indicated by the scale bar. The tree showing the evolutionary relationships between these genes was constructed using TreeView.

Specifically, the null hypothesis of no recombination was clearly rejected with $P < 0.001$. In an attempt to pinpoint the sequences involved, we used maxchi2, methods from the TOPALi program and BLAST searches on several different subsets of the full-length alignment. These investigations confirmed that subgroup III sequences could well be the result of recombination between subgroup I and subgroup II sequences: maxchi2 and TOPALi analyses on subsets containing representatives from all three groups often gave significant results, and BLAST often found one part of a subgroup III sequence to be more similar to subgroup I sequences while the other part was more similar to sequences belonging to subgroup II.

However, it should be noted that the picture was not quite this simple, as some analyses also found significant evidence of recombination not just between, but also within, subgroups I and II.

## Discussion

Phylogenetic analysis divided the *tet*(M) genes into three major subgroups based on both partial and full-length sequences (Figure 2). Interestingly, *tet*(M) genes from isolates with Tn*5397*-like elements clustered with the Tn*5397*-associated *tet*(M) from *C. difficile* (AF333235) in subgroup I (Figure 2). Moreover, these *tet*(M) genes were clearly different from *tet*(M) in Tn*916*-like elements found in subgroup II (Figure 2).

Two *E. faecium* isolates from pig and broiler, respectively (9830470-4 and 9830409-1), had *tet*(M) present on 80 kb plasmids (data not shown). The *tet*(M) genes from these isolates branched separately in subgroup I (Figure 2). We could not detect transposons in these isolates, and no horizontal transfer of *tet*(M) from these isolates to *E. faecalis* (JH2-2) or *E. faecium* (BM4105) was observed. One or more plasmids were detected in 8 of the 17 isolates, but since the *tet*(M) probe hybridized to plasmids in only 2 isolates, *tet*(M) was present either on the chromosome or on large plasmids (>150 kb, not extracted by this method) in the majority of isolates (data not shown).

Of the isolates with *tet*(M) on Tn*916*/Tn*1545* family transposons, 11 were sequenced. Of these isolates, 10 were grouped into subgroup II and 8 were 100% identical to *tet*(M) from Tn*916* found in *E. faecalis* (GenBank accession nos. M85225 and U09422) and were isolated from *E. faecalis* from pigs and broilers and from *E. faecium* from humans and pigs (Figure 2). Partial sequences of *tet*(M) from *Enterococcus durans* (GenBank accession no. AJ585084) and *Staphylococcus aureus* (GenBank accession no. AY057893) were also identical (data not shown). The 100% identical *tet*(M) in these isolates may indicate either recent transfer events or that no adaptation of the *tet*(M) has been necessary in order for the gene to be maintained in these species. Subgroup II contains several *tet*(M) genes associated with Tn*916*-like elements. For instance, a group within subgroup II includes *E. faecalis* and *Bacillus cereus* with transferable *tet*(M) on Tn*916*/Tn*1545* family transposons located on the main chromosome. These bacteria were isolated from shellfish and farmland soil, respectively. However, this group also contains *tet*(M) associated with an element designated CW459*tet*(M), which contains the integrase *int459* from *Clostridium perfringens*. Furthermore, the majority of *tet*(M) genes in this group are plasmid borne and often show no indication of the presence of any Tn*916*/Tn*1545* family transposon.[7] Even though the mobile elements carrying *tet*(M) seem very diverse in subgroup II and are from both Gram-positive and Gram-negative origin, the majority of *tet*(M) genes are highly related, with zero or a few base pair differences.

One *E. faecalis* isolate from human had highest homology to *tet*(M) on Tn*1545* from *E. faecalis* (GenBank accession no. X04388) within subgroup III. This isolate had *erm*(B) and *aphA-3* responsible for resistance to erythromycin and kanamycin, respectively, and these genes were present on Tn*1545*. This strain may contain *tet*(M) on a Tn*1545*-like transposon rather than on a Tn*916*-like transposon.

Mosaic structures resulting from recombination within the *tet*(M) group were observed by Oggioni *et al*.[24] in *E. faecalis*, *S. pneumoniae*, *S. aureus*, *Ureaplasma urealyticum* and *Neisseria*, and they could be traced to two distinct alleles. Huang *et al*. found mosaic structures within *tet*(M) of *Gardnerella* with regions of homology to *tet*(M) gene sequences from Tn*916/Tn1545* and the American type plasmid found in *Neisseria gonorrhoeae*.[25] Recombination within the *tet*(M) group was also confirmed by our study. Subgroup III was peculiar not only in consisting of several *tet*(M) genes located on composite transposons (Tn*1545*, Tn*2009* and Tn*5251*) but also in being placed more closely to the root and having relatively low clade-credibilities. These features could indicate that some or all subgroup III sequences are the result of recombination between subgroup I and/or II sequences; we therefore used a variety of different tools to investigate this hypothesis further. Significant evidence for recombination not just between but also within subgroups II and I was found. For recombination to take place between *tet*(M) genes on different mobile elements, both elements must be present within the same cell. We found in this study one *E. faecium* isolate with both Tn*916*/Tn*1545*-like and Tn*5397* transposons. This is to our knowledge the first finding of *tet*(M) on two different mobile elements within the same isolate, and this supports the notion that recombination between *tet*(M) genes is possible. This finding is also interesting since studies of these two transposons within the same *C. difficile* cell have shown that Tn*5397* can induce the loss of Tn*916* when introduced into a *C. difficile* cell by conjugation and vice versa.[6] The Tn*5397* had an insert of ∼1500 bp downstream to the *tet*(M) gene, and the circular form of the transposon could not be detected and horizontal transfer of the transposon could not be demonstrated, indicating that the transposon was not functional, which could explain the presence of both transposons within the same isolate. However, this needs to be further studied.


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## Transparency declarations

None to declare.

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