Prevalence and Molecular Characterization of Tetracycline Resistance in *Enterococcus* Isolates from Food

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In the present study, a collection of 187 *Enterococcus* food isolates mainly originating from European cheeses were studied for the phenotypic and genotypic assessment of tetracycline (TC) resistance. A total of 45 isolates (24%) encompassing the species *Enterococcus faecalis* (*n* = 33), *E. durans* (*n* = 7), *E. faecium* (*n* = 3), *E. casseliflavus* (*n* = 1), and *E. gallinarum* (*n* = 1) displayed phenotypic resistance to TC with MIC ranges of 16 to 256 μg/ml. Eight of these strains exhibited multiresistance to TC, erythromycin, and chloramphenicol. By PCR detection, TC resistance could be linked to the presence of the *tet*(M) (*n* = 43), *tet*(L) (*n* = 16), and *tet*(S) (*n* = 1) genes. In 15 isolates, including all of those for which the MIC was 256 μg/ml, both *tet*(M) and *tet*(L) were found. Furthermore, all *tet*(M)-containing enterococci also harbored a member of the Tn*916*-Tn*1545* conjugative transposon family, of which 12 erythromycin-resistant isolates also contained the *erm*(B) gene. Filter mating experiments revealed that 10 *E. faecalis* isolates, 3 *E. durans* isolates, and 1 *E. faecium* isolate could transfer either *tet*(M), *tet*(L), or both of these genes to *E. faecalis* recipient strain JH2-2. In most cases in which only *tet*(M) was transferred, no detectable plasmids were acquired by JH2-2 but instead all transconjugants contained a member of the Tn*916*-Tn*1545* family. Sequencing analysis of PCR amplicons and evolutionary modeling showed that a subset of the transferable *tet*(M) genes belonged to four sequence homology groups (SHGs) showing an internal homology of ≥99.6%. Two of these SHGs contained *tet*(M) mosaic structures previously found in Tn*916* elements and on *Lactobacillus* and *Neisseria* plasmids, respectively, whereas the other two SHGs probably represent new phylogenetic lineages of this gene.

As lactic acid bacteria, enterococci are natural inhabitants of the gastrointestinal systems of mammals, but they are also known to occur in soil and fecally polluted surface waters and on plants and vegetables (26, 31, 35). Because of their high prevalence in the gastrointestinal tracts of many food animals, it is often unavoidable that these organisms enter the human food chain via contamination of raw milk or raw meat. For many years, the presence of enterococci in foods has been highly controversial. On the one hand, *Enterococcus* strains can harbor specific biochemical traits that are essential in the manufacturing of various fermented milk products, and some strains are technologically exploited as functional starters or probiotics (12, 17). On the other hand, enterococci have also been implicated in the spoilage of processed meats (14, 51) and include strains that have been recognized as emerging human pathogens mostly in nosocomial but also in community-acquired infections (for a review, see reference 29).

Triggered by the apparent duality between their beneficial and harmful properties, a lot of research has focused on the potential role of food enterococci as reservoirs and/or vehicles of antibiotic resistance (AR) and virulence factors (12, 24, 25) and on the possible interaction between AR and virulence (28). During the antibiotic era, an increasing number of food enterococci have developed resistance to various therapeutic antibacterial agents, including vancomycin (18, 40, 52), genta-

micin (11), and streptogramines (42). From studies in which patterns of AR to a broader range of therapeutic agents were determined, it appears that tetracycline (TC) resistance (Tc[r]) is one of the most common phenotypes of acquired AR in food isolates of the genus *Enterococcus* (34, 45). Although the broad-spectrum clinical use of TCs is declining, new applications of minocycline and glycylcycline in human therapy have been identified (7). Moreover, this group of agents is still used in veterinary and aquaculture settings and some TCs are still in use as animal growth promoters in several countries outside Europe (7).

In enterococci, two major groups of Tc[r] (*tet*) genes have been recognized (7). The first group confers resistance by ribosomal protection (RP) and includes the genes *tet*(M), *tet*(O), and *tet*(S), which have been detected in *Enterococcus* spp. A second group mediates energy-dependent efflux of TC from cells and is represented in enterococci by the *tet*(K) and *tet*(L) genes. A sixth gene, *tet*(U), encodes low-level resistance in *Enterococcus faecium* through an unknown mechanism (39). So far, the great majority of studies dealing with the distribution of *tet* genes in *Enterococcus* spp. have focused on human or animal isolates. In contrast, data on the occurrence of *tet* genes in *Enterococcus* isolates obtained from food and food products are still relatively scarce. Teuber and coworkers (45, 46) reported the presence of *tet*(M) in six *Enterococcus* isolates from cheese and salami, some of which were located both on the chromosome and on a large plasmid. In one isolate, *E. faecalis* FO1, the *tet*(M) gene was located on a Tn*916*-like transposon named Tn*FO1* (33).

Of the six types of *tet* genes detected in veterinary or clinical

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enterococci, only *tet*(M) has been reported in *Enterococcus* isolates from food. To obtain further insight into the prevalence and diversity of *tet* genes among food enterococci, the present study pursued the phenotypic and genotypic assessment of Tc^r among a collection of taxonomically well-characterized *Enterococcus* isolates originating mainly from cheeses but also from other food sources. In addition, a subset of *tet* genes belonging to the *tet*(M) group that could be transferred in vitro by filter conjugation to *E. faecalis* recipient strain JH2-2 was further characterized by partial sequencing to examine their degree of nucleotide polymorphism and their sequence similarities to previously determined *tet*(M) genes.

MATERIALS AND METHODS

Bacterial isolates. The 187 *Enterococcus* isolates included in this study were obtained from the BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium (http://www.belspo.be/bccm/db/bacteria_search.htm), and all originated from food sources. The majority of the isolates (*n* = 139) were isolated from different types of European cheeses (mostly from Italy, Ireland, and Greece) that were obtained in the course of European Union research project FAIR-CT97-3078 (Enterococci in Food Fermentations: Functional and Safety Aspects). More information on these isolates can be found in the catalogue of enterococci of the FAIR-E collection (50). All of the enterococci included in this study were previously identified to the species level by protein profiling. Isolates were preserved with the Microbank bead storage system (Pro-LAB Diagnostics, Wirral, United Kingdom) at −80°C and routinely grown on MRS agar (CM359; Oxoid, Basingstoke, United Kingdom) at 30 or 37°C under aerobic conditions.

Determination of phenotypic resistance. First, all 187 isolates were screened for the Tc^r phenotype by an agar dilution method. Cultures grown overnight were inoculated on MRS plates containing TC at 25 μg/ml (T-3383; Sigma, St. Louis, Mo.), and growth was scored visually after 24 to 72 h. All isolates that displayed the Tc^r phenotype after agar dilution were subjected to MIC testing in the range of 4 to 512 μg of TC per ml based on the broth microdilution method with cation-adjusted Mueller-Hinton II broth (212322; Becton Dickinson, Cockeysville, Md.) as recommended by the National Committee for Clinical Laboratory Standards (NCCLS; 30). Following a 24-h incubation, the MIC was visually read from a microtiter plate as the concentration at which ≥80% inhibition of growth occurred. Each batch of MIC determinations included the control strain *E. faecalis* LMG 8222 (ATCC 29212).

The presence of additional phenotypic resistances was determined by the agar disk diffusion method as previously described (30). Antibiotic disks (Oxoid) containing TC (30 μg), doxycycline (30 μg), minocycline (30 μg), ampicillin (10 μg), rifampin (30 μg), vancomycin (30 μg), erythromycin (ER, 15 μg), and chloramphenicol (CM; 30 μg) were used. Inhibition zones were interpreted in accordance with the NCCLS guideline tables (30).

Detection of AR and *int* genes. Total genomic DNA was prepared by using a protocol based on the method of Pitcher and coworkers (36). Isolation of plasmid DNA was based on conventional alkaline lysis (2).

For all detection assays, a common PCR core mixture (total volume, 50 μl) was used that consisted of 1× PCR buffer (Applied Biosystems, Warrington, United Kingdom), deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Applied Biosystems) at a concentration of 200 μM each, 1 U of AmpliTaq DNA polymerase (Applied Biosystems), and 20 pmol of each primer (Sigma-Genosys Ltd., Cambridgeshire, United Kingdom). A 50-ng portion of intact total DNA was used as the PCR template. All genes were detected with previously described primers and PCR control strains. In a first PCR assay, *tet* genes encoding RP proteins were detected with the primers DI and DII (15) and primers Ribo2-FW and Ribo2-RV (1). Isolates harboring an RP protein-type *tet* gene were further subjected to PCR detection of *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), and *tet*(W) with primers DI and TetM-R, primers TetO-FW1 and TetO-RV1, primers TetQ-FW and TetQ-RV, primers TetS-FW and TetS-RV, primers TetT-FW and TetT-RV, and primers TetW-FW and TetW-RV (1, 15), respectively. The presence of the efflux genes *tet*(K) and *tet*(L) was determined with primers TetK-FW1 and TetK-RV1 and primers TetL-FW3 and TetL-RV3, respectively (15). The presence of the *erm*(B) gene was investigated with primers ErmB-FW and ErmB-RV (15). The occurrence of conjugative transposons of the Tn*916*-Tn*1545* family was determined with primers Int-FW and Int-RV targeting the transposon integrase (*int*) gene (10, 15). All PCR amplifications were performed in a GeneAmp 9600 PCR system (Perkin-Elmer) with the temperature

program previously described for each primer set (1, 15). PCR amplicons were checked electrophoretically on 1% agarose and visualized by ethidium bromide fluorescence.

Filter matings. All strains harboring one or more *tet* genes were included in filter matings with *E. faecalis* strain JH2-2 (21) on the basis of the protocol described by Perreten and coworkers (33). In short, 1 ml of a culture of the donor or recipient strain grown overnight in brain heart infusion (BHI) broth (Difco) at 37°C was added to 5 ml of fresh BHI broth and further incubated for 4 h. Equal volumes (1 ml) of donor and recipient cultures were mixed and filtered through a sterile membrane filter with a pore size of 0.45 μm (MF-Millipore membrane filter HAWP 2500; Millipore, Bedford, Mass.) contained in a Swinnex filter holder (SX00 02500; Millipore). Subsequently, filters were gently rinsed once with 2 ml of a sterile peptone-physiological saline (PPS) solution (8.5 g of NaCl per liter, 1g of neutralized bacteriological peptone [Oxoid L34] per liter). Filters were incubated on BHI agar (Difco) for 24 h at 37°C, and after mating, cells were washed from the filter with 2 ml of PPS. Finally, serial dilutions in PPS of the mixed suspension and of the donor and recipient strains were plated on BHI agar supplemented with 10 μg of TC per ml, 50 μg of rifampin per ml, and 100 μg of fusidic acid per ml (triple selective medium). The TC-susceptible JH2-2 recipient strain and the potential donor strains are resistant and susceptible, respectively, to the latter two antibiotics at the indicated concentrations. Following incubation at 37°C for 24 to 72 h, plates were checked for the absence (donor and recipient plates) or presence (mating mixture plate) of growth and up to 10 colonies were picked from the latter plate and inoculated into BHI broth supplemented with 10 μg of TC per ml. Upon growth, potential transconjugant cultures were purified on BHI agar and stored with the Microbank bead storage system at −80°C. Phenotypic and genotypic properties of possible transconjugants were determined by disk diffusion susceptibility testing, determination of the MIC of TC, (GTG)$_5$-PCR DNA fingerprinting (16), plasmid profiling, and PCR-based detection of *tet* genes and other resistance elements as described above.

Sequencing of PCR amplicons. A subset of PCR amplicons of *tet*(M) genes detected in JH2-2 transconjugants were purified and sequenced with primers DI, DII, and TetM-R (15). As a sequencing control, the sequence of the *tet*(M) gene of strain *E. faecalis* FO1 (EMBL accession no. X92947) (35) was partially redetermined. Sequencing was performed with a BigDye Terminator (version 2) Ready Reaction cycle sequencing kit (Applied Biosystems) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Reference sequences of *tet*(M) were retrieved from the EMBL database (http://www.ebi.ac.uk) and compared with the new sequences by using BioNumerics version 3.5 software (Applied Maths, St.-Martens Latem, Belgium). Additional sequence similarity searches were performed with the EMBL Fasta program.

Nucleotide sequence accession numbers. The partial sequences of the *tet*(M) genes described in this paper have been submitted to the EMBL database under accession numbers AJ585076 to AJ585084.

RESULTS AND DISCUSSION

Prevalence of Tc^r among food enterococci. In order to increase our understanding of the molecular ecology and population biology of Tc^r determinants and their hosts, surveillance and characterization of Tc^r should not only be restricted to pathogenic bacteria but should also include organisms from other environmental niches, such as food-associated and commensal bacteria (43; http://www.tufts.edu/med/apua/ROAR /roarhome.htm). In this regard, enterococci constitute an interesting group as they are commensals of humans and animals that occur and grow in a variety of foods and have also been implicated in nosocomial infections. In the present survey, a collection of 187 enterococcal isolates, mostly from cheese but also from other food sources (Table 1), were studied for the presence of Tc^r, of which 45 isolates (24%) displayed the Tc^r phenotype on the basis of agar dilution testing with a breakpoint concentration of 25 μg of TC per ml. The majority of the Tc^r isolates belonged to the species *E. faecalis* (*n* = 33) and *E. durans* (*n* = 7), whereas only a few Tc^r isolates of *E. faecium* (3 out of 55) were found (Table 1). Of the remaining species, only *E. casseliflavus* and *E. gallinarum* both yielded one Tc^r isolate,

TABLE 1. Prevalence of the Tc^r phenotype in *Enterococcus* isolates from food

Type(s) of food	No. of Tc^r isolates/total (% above arbitrary threshold)						
	E. casseliflavus	*E. durans*	*E. faecalis*	*E. faecium*	*E. gallinarum*	Other species[a]	Total
Cheese	1/2	6/21 (29)	24/65 (37)	2/39 (5)	1/5	0/7	34/139 (24)
Milk	0/1	0/1	3/9	0/3		0/1	3/15 (20)
Meat and meat products			1/4	0/6			1/10
Fish		1/1	2/5				3/6
Vegetables			0/1	0/3		0/1	0/5
Fermented milk		0/1	2/2				2/3
Others			1/4	1/4		0/1	2/9
Total	1/3	7/24 (29)	33/90 (37)	3/55 (5)	1/5	0/10	45/187 (24)

[a] Isolates of *E. hirae* ($n = 7$), *E. malodoratus* ($n = 2$), and *E. flavescens* ($n = 1$).

but clearly more isolates should be included in order to assess the prevalence of the Tc^r phenotype in these taxa. Most of the Tc^r *Enterococcus* isolates originated from cheese ($n = 34$), followed by a few Tc^r isolates from milk, fermented milks, and fish, among other food sources (Table 1). The relative prevalence of Tc^r enterococci encountered in this study (24%) falls within the range of a previous estimate of 20 to 97% Tc^r among enterococci from animals and their meat (45). However, it should be stressed that the prevalence of Tc^r among enterococcal isolates from food can vary significantly, depending on the type of food product (5), geographical origin (37), and taxonomic identity (13). The recovery of only a small minority of Tc^r *E. faecium* strains in our study (5%) may be due to the fact that most of the isolates originated from cheese. Considerably higher percentages of Tc^r *E. faecium* isolates (28 to 74%) can be expected when enterococci from retail poultry are investigated (5, 37). Some of the isolates used in our study were also previously included for an E-test analysis (13), in which it was found that *E. faecium* displayed fewer resistance phenotypes than *E. faecalis* among cheese isolates.

For all 45 Tc^r *Enterococcus* isolates, the minimum TC MIC was 16 μg/ml, which is the cutoff level proposed by the NCCLS (30) for the classification of enterococci as Tc^r. For *E. faecalis* strains, MICs ranged from 16 to 256 μg/ml, whereas a somewhat lower range of 32 to 128 μg/ml was found for the Tc^r *E. durans* and *E. faecium* strains (Table 2). Susceptibilities to additional antibiotics were assessed by disk diffusion testing by the NCCLS method with Mueller-Hinton agar (30), which was compared with the previously reported agar overlay method using MRS agar (20). In terms of interpretive zone reading, the

data sets generated by both methods were compatible, with the exception of inhibition zones for ER, which were occasionally smaller on MRS agar (results not shown). The Tc^r phenotype was uniformly confirmed by disk diffusion testing, and all Tc^r isolates also displayed resistance to other TCs, i.e., doxycycline and minocycline, but were susceptible to ampicillin, rifampin, and vancomycin (data not shown). In addition, about one-third of the Tc^r *E. faecalis* isolates were coresistant to ER (12 of 33 strains) and/or CM (10 of 33 strains) (Table 2), of which 8 displayed multiple resistance to TC, ER, and CM. Teuber and coworkers (45) indicated that resistance to TC, ER, CM, and gentamicin was a common multiple drug resistance phenotype displayed by *Enterococcus* isolates from various types of cheeses.

Prevalence of *tet* genes and other resistance elements. Various studies on the antibiotic susceptibilities of human enterococci obtained in the early 1950s indicate that a significant pool of Tc^r determinants was already prevalent in clinical isolates during the first years of therapeutic use of TC (3, 23, 48). In the present study, the genotypic basis of the Tc^r phenotype found in food isolates of *Enterococcus* spp. was investigated by PCR-based detection of eight *tet* genes, all of which, except *tet*(T), *tet*(Q), and *tet*(W), have been previously reported in this genus (7). As shown in Table 2, the *tet*(M) gene was found in all but two Tc^r *Enterococcus* isolates and one *E. faecalis* strain contained *tet*(S) whereas none of the other RP genes tested for [*tet*(O), *tet*(Q), *tet*(T), or *tet*(W)] were detected. Of the efflux genes *tet*(K) and *tet*(L), only the latter was detected in 16 Tc^r isolates mainly belonging to the species *E. faecalis* and in 1 Tc^r *E. durans* isolate. The sole Tc^r *E. casseliflavus* isolate encoun-

TABLE 2. Phenotypic and genotypic resistance properties of Tc^r food enterococci[a]

Species	No. of isolates	TC MIC (μg/ml)	Additional resistance[b]			*tet*(M)	*tet*(L)	*tet*(S)	Tn*916*–Tn*1545*	*erm*(B)	Transfer to JH2-2
			ER + CM	ER	CM						
E. casseliflavus	1	128	0	0	0	0	0	0	0	0	0
E. durans	7	32–128	0	1	0	7	1	0	7	1	3
E. faecalis	33	16–256	8	2	2	32	13	1	30	10	10
E. faecium	3	32–128	0	1	0	3	2	0	3	1	1
E. gallinarum	1	64	0	0	0	1	0	0	1	0	0
Total	45		8	4	2	43	16	1	41	12	14

[a] Unless noted otherwise, each value is the number of isolates with the property indicated.
[b] As determined by disk diffusion.

tered in this study did not contain any of the above-mentioned *tet* genes. For this isolate, originating from smear-ripened raw milk cheese, the MIC was 128 µg/ml, which indicates that it contains an unknown mechanism conferring high-level Tc^r. The finding that all Tc^r isolates harbored an RP *tet* gene is concordant with the minocycline resistance phenotype found by disk diffusion testing, which is mediated only by RP mechanisms in enterococci (7). A similar situation was found during a major survey of 229 enterococcal isolates collected in 10 hospitals in France, where *tet*(M) and *tet*(L) were the dominant Tc^r determinants, followed by *tet*(S), whereas *tet*(O) was found in only 1 isolate (6). It should be kept in mind that the *tet* gene distribution reported in this study is based on a relatively limited set of isolates, which highlights the need for regular monitoring of additional sets of biologically and geographically diverse strains in order to obtain a broader view of the prevalence of these genes in food-associated enterococci. Except for one strain, all of the strains in our study that were found to contain *tet*(L) also possessed the *tet*(M) gene. It is noteworthy that the six *E. faecalis* strains for which the MIC of TC was the highest (256 µg/ml) contained both *tet*(M) and *tet*(L), whereas for the sole strain that harbored only *tet*(L) and no RP gene, the MIC was the lowest observed in this study (16 µg/ml). This observation suggests that a higher level of Tc^r is reached when the organism contains both an active-efflux mechanism and an RP mechanism. Such a reinforcement effect has previously also been reported for methicillin-resistant *Staphylococcus aureus* (49), for isolates containing either *tet*(M) or *tet*(K) the TC MIC for 90% of the strains tested was 64 µg/ml whereas for those that contained both of these *tet* genes the TC MIC for 90% of the strains tested was 256 µg/ml.

In many enterococci and streptococci of clinical or food origin, drug resistance genes occur more frequently on conjugative transposons than on plasmids. Also in this study, the majority of the *tet*(M)-containing isolates (41 of 43 isolates) were positive by PCR for the integrase element *int*, indicating that they contain a member of the broad-host-range Tn*916*-Tn*1545* conjugative transposon family (Table 2). By definition, all members of this family carry the *tet*(M) gene, sometimes joined by additional determinants, e.g., those encoding resistance to ER and kanamycin in the case of Tn*1545* (8). In our study, the 10 *E. faecalis* isolates and single isolates of *E. durans* and *E. faecium* that were Er^r all contained *erm*(B), which is considered to be the most widespread macrolide resistance gene among enterococci from food animals (22) or foods (45). Furthermore, all of these *erm*(B)-containing strains were also positive for the detection of a Tn*916*-Tn*1545* element. In this context, it should be mentioned that *erm*(B) genes in enterococci can also occur on other mobile elements, such as conjugative multiresistance plasmids (47) or members of the Tn*917* family (41).

Conjugal transfer of Tc^r. The historical spread of Tc^r into and among enterococcal populations, including those from food environments, has been strongly associated with the fact that many *tet* genes are located on mobile genetic elements (45). On the basis of the finding that most of the Tc^r isolates in the present study also carried a member of the Tn*916*-Tn*1545* family (Table 2) or harbored one or more plasmids (data not shown), we investigated the mobility of the detected *tet* genes in filter mating experiments with TC-susceptible recipient

strain *E. faecalis* JH2-2 (21). Initially, potential transconjugant colonies were obtained from 18 out of 45 tested Tc^r donor strains representing the species *E. faecalis*, *E. durans*, and *E. faecium*. However, after phenotypic and genotypic confirmation, transconjugants of four *E. faecalis* isolates were found to be susceptible to rifampin and/or displayed (GTG)$_5$-PCR fingerprints highly similar, if not identical, to those of the respective donors. As a result, the number of successful matings with strain JH2-2 was reduced to 14 (31% of analyzed donors), all of which were obtained from donor strains originating from Italian or Irish cheeses ($n = 12$), milk ($n = 1$), or shellfish ($n = 1$) (Table 3). Overall, transfer frequencies obtained during filter matings were in the range of 10^{-6} to 10^{-8} per recipient.

For transconjugants obtained from 10 *E. faecalis* donors, the TC MIC was equal to or lower than that for the corresponding donor, except for isolates LMG 20678, LMG 20693, LMG 20799, and LMG 20831, for which the MICs were 1 or 2 doubling concentrations higher (Table 3). For transconjugants obtained from three *E. durans* isolates, the MICs were consistently lower than those for the donors, whereas for a set of transconjugants obtained from *E. faecium* LMG 20927, the MICs did not exceed 128 µg/ml. Because of the limited number of Tc^r donors included, it is not clear to what extent these differences in MICs may reflect increased or decreased levels of *tet* gene expression. For matings with *E. faecalis* donors, Tc^r in JH2-2 transconjugants was linked to acquisition of the *tet*(M) gene ($n = 8$), the *tet*(L) gene ($n = 1$), or both of these genes ($n = 1$). All transconjugants from *E. durans* acquired *tet*(M) and, in contrast, transconjugants from *E. faecium* LMG 20927 harbored either *tet*(M) or both *tet*(M) and *tet*(L), which explains the relatively broad MIC range found for this mating (Table 3). One *E. faecalis* donor and one *E. faecium* donor (LMG 20790 and LMG 20927, respectively), both carrying an *erm*(B) gene, could transfer this gene to recipient strain JH2-2, whereas three other *E. faecalis* donors cotransferred Cm^r (Table 3). To our knowledge, conjugal transfer of TC, ER, and/or CM resistance by food enterococci has previously only been reported by Teuber and colleagues (46) for isolates from cheese and meat products. These authors reported that Tc^r acquisition by JH2-2 was due to transfer of the *tet*(M) gene, which in some cases was located both on the chromosome and on a large plasmid (46). Our study provides the first indication that also *tet*(L), sometimes in combination with *tet*(M), can be transferred in vitro by food isolates of *E. faecalis* and *E. faecium*. For three donors, transfer of *tet*(L) was associated with the acquisition of one or more large plasmids (>20 kb). In most other cases (8 out of 11 matings), in which only *tet*(M) was transferred to recipient JH2-2, no plasmid transfer was detected (data not shown) but all transconjugants contained a representative of the Tn*916*-Tn*1545* family (Table 3). Members of this family have been found in more than 50 different bacterial species, most of which are of clinical importance (8). Our data thus seem to reinforce the evidence that many enterococci, regardless of having a clinical background or a food origin, use conjugative transposition as an important mechanism for the dissemination of *tet*(M) and possibly other resistance genes.

Genetic diversity of *tet*(M) genes from JH2-2 transconjugants. The finding that the same types of *tet* genes, such as *tet*(M), occur in veterinary, clinical, and food enterococci raises

TABLE 3. Phenotypic and genotypic resistance properties of *Enterococcus* donors and resulting JH2-2 transconjugants from filter mating experiments

Donor strain (FAIR-collection no.)	Biologic origin	Geographic origin (yr of isolation)	TC MIC (µg/ml)		Additional resistance(s)		*tet* gene(s)		Tn916-Tn1545		*erm*(B)		No. of plasmids transferred (size[s] of plasmid[s] [kb])	*tet*(M) SHG
			Donor	Trans-conjugant	Donor	Trans-conjugant	Donor	Trans-conjugant	Donor	Trans-conjugant	Donor	Trans-conjugant		
E. durans														
LMG 20930 (FAIR-E 387)	Casera cheese	Italy (1980)	128	64			*tet*(M)	*tet*(M)	+	+	−	−	Not detected	
LMG 20931 (FAIR-E 388)	Casera cheese	Italy (1980)	128	64			*tet*(M)	*tet*(M)	+	+	−	−	Not detected	
LMG 20932 (FAIR-E 389)	Valtellina cheese	Italy (1981)	128	64			*tet*(M)	*tet*(M)	+	+	−	−	Not detected	III
E. faecalis														
LMG 20647 (FAIR-E 42)	Shellfish	Italy (1997)	64	64	ER		*tet*(M)	*tet*(M)	+	+	−	−	Not detected	I
LMG 20678 (FAIR-E 74)	Montasio cheese	Italy (1997)	32	128	ER		*tet*(M)	*tet*(M)	+	+	−	−	Not detected	V
LMG 20693 (FAIR-E 90)	Goat cheese	Italy (1997)	32	64	ER		*tet*(M)	*tet*(M)	+	+	−	−	Not detected	V
LMG 20786 (FAIR-E 224)	Cheddar cheese	Ireland (1994)	64	64	ER + CM	CM	*tet*(M), *tet*(L)	*tet*(M)	+	+	+	−	Not detected	IV
LMG 20790 (FAIR-E 228)	Cheddar cheese	Ireland (1994)	64	64	ER	ER	*tet*(M)	*tet*(M)	+	+	+	+	Not detected	IV
LMG 20791 (FAIR-E 229)	Cheddar cheese	Ireland (1994)	256	32	ER + CM	CM	*tet*(M), *tet*(L)	*tet*(L)	+	−	−	−	1 (>20)	
LMG 20798 (FAIR-E 236)	Smear-ripened cheese	Ireland (1994)	256	64	CM	CM	*tet*(M), *tet*(L)	*tet*(M), *tet*(L)	+	+	−	−	2 (>20)	IV
LMG 20799 (FAIR-E 237)	Cheddar cheese	Ireland (1995)	32	64	ER		*tet*(M), *tet*(L)	*tet*(M)	+	+	−	−	3 (4.8, 5, >20)	
LMG 20831 (FAIR-E 275)	Milk	Italy (1997)	32	32-64			*tet*(M)	*tet*(M)	+	+	−	−	5 (4.8, 5, 9.8, 20, >20)	III
LMG 20843 (FAIR-E 287)	Fontina cheese	Italy (1997)	128	64	ER		*tet*(M)	*tet*(M)	+	−	−	−	2 (5, 9.8)	III
E. faecium LMG 20927 (FAIR-E 384)	Grana cheese	Italy (1976)	128	32–128	ER	ER	*tet*(M), *tet*(L)	*tet*(L)/*tet*(M), *tet*(L)[a]	+	+	+	+	3 (4.7, 5.3, >20)[a]	III

[a] Transconjugants of LMG 20927 acquired either *tet*(L) or both *tet*(M) and *tet*(L). Plasmid profiles of these transconjugants differed in the number of acquired plasmids.
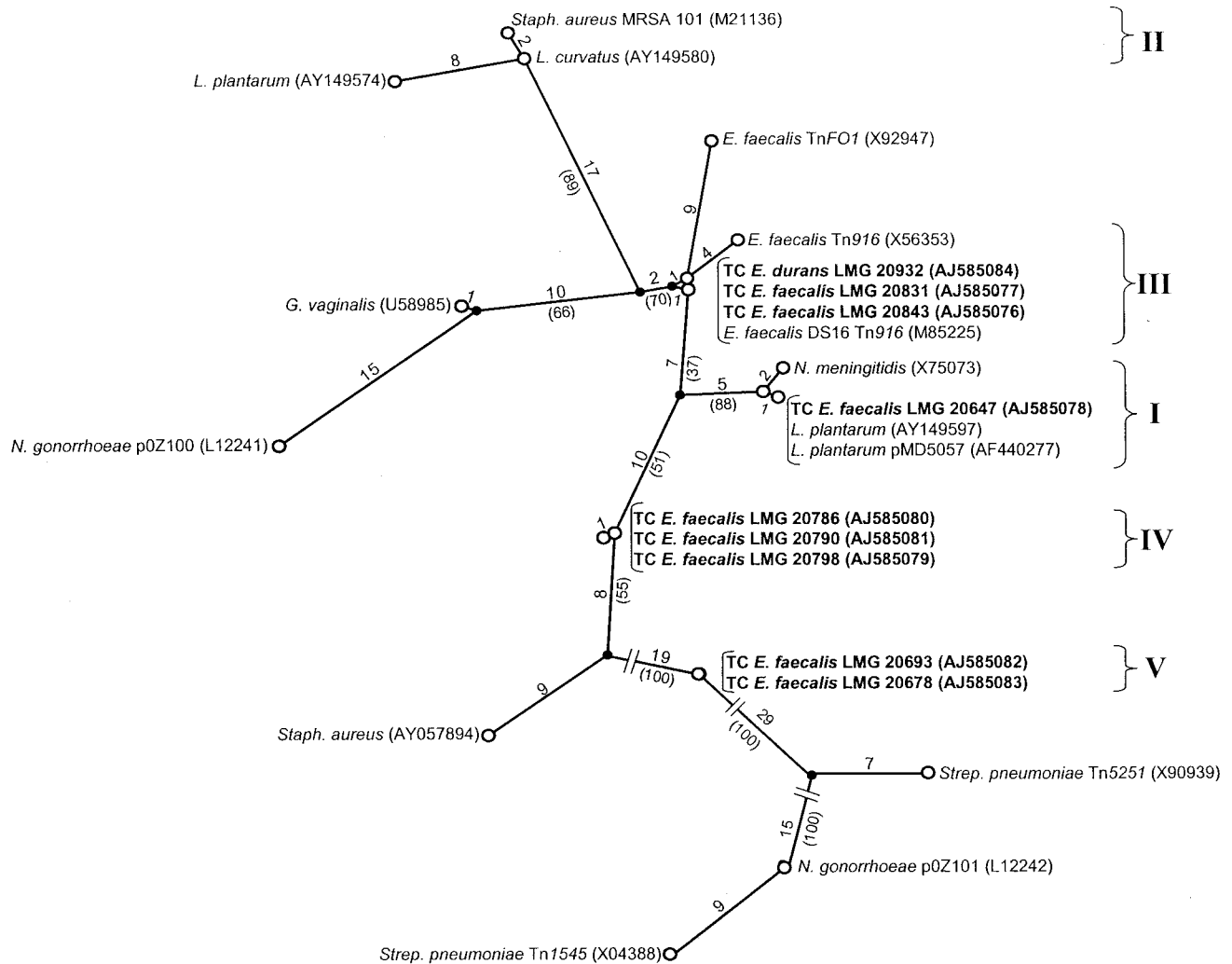
FIG. 1. Unrooted maximum-parsimony tree of multiple aligned partial *tet*(M) sequences of enterococcal JH2-2 transconjugants and reference *tet*(M) sequences retrieved from the EMBL database. SHGs showing ≥99.6% internal sequence homology are indicated by roman numerals I to V. The number of base conversions over the tree is indicated along the phylogenetic distance lines, and bootstrap percentages for analysis of 100 replicates are in parentheses. If available, the designation of the *tet*(M)-carrying strain, plasmid, or transposon is indicated, followed by the EMBL accession number in parentheses. Newly obtained *tet*(M) sequences are in boldface.

the question of the extent to which genetic transfer and/or recombination events have played a role in the global distribution of such genes. On the basis of gene sequence alignments (19, 32) or high-resolution gene restriction analysis (10), several authors have shown that the *tet*(M) gene exhibits several mosaic structures, possibly as a result of a series of homologous recombinations. To examine if any of these previously observed mosaic structures also occur in transferable *tet*(M) genes of food enterococci, a subset of *tet*(M) genes that could be transferred by nine donor isolates to recipient strain JH2-2 was further analyzed by sequencing of the DI–TetM-R PCR amplicons obtained from the corresponding transconjugants. Although it can be expected that sequence analysis of the complete *tet*(M) gene will result in a more robust evolutionary model, analysis of amplicon sequences that encompass 74% of the 1,920-bp coding sequence already provides a reliable indication of the phylogenetic diversity among enteroco-

cal *tet*(M) genes. Global alignment and evolutionary modeling by maximum-parsimony analysis allowed us to group these sequences into four different sequence homology groups (SHGs) compared with reference *tet*(M) sequences retrieved from the EMBL database (Fig. 1). The delineation of these SHGs was based on an internal sequence homology level of ≥99.6%, which generally corresponded to zero to six nucleotide substitutions. Concordant with a previously proposed nomenclature in which two *tet*(M) SHGs (I and II) were defined by maximum-parsimony analysis (15), the newly obtained *Enterococcus tet*(M) sequences were assigned to SHGs I, III, IV, and V, whereas no new members of SHG II were found. In addition to the five currently defined SHGs, at least seven other *tet*(M) structures have previously been recognized, some of which may represent additional SHGs (Fig. 1).

SHG I comprised one new *tet*(M) sequence of *E. faecalis* LMG 20647, which was joined by *tet*(M) genes previously

found on plasmids in *Neisseria meningitidis* (accession no. X75073) and only recently also in lactobacilli (9, 15). Although SHG I contained *tet*(M) sequences originating from different gram-positive and gram-negative genera, all of these sequences were very highly related, showing only zero to three nucleotide variations. Transfer of *tet*(M) from isolate LMG 20647 to recipient JH2-2 was not accompanied by the acquisition of one or more detectable plasmids, suggesting that this transfer may be mediated by a conjugative transposon element of the Tn*916*-Tn*1545* family, as indicated by the presence of an *int* gene in the resulting transconjugants. SHG III included *tet*(M) genes from *E. faecalis* LMG 20831 and LMG 20843 and *E. durans* LMG 20932 and also comprised the prototype mosaic structure of the *tet*(M) gene located within conjugative transposon Tn*916* (4, 44). The partial *tet*(M) gene of the three Italian *Enterococcus* isolates displayed only zero to two nucleotide differences from each other or from the *tet*(M) sequence of Tn*916*-carrying *E. faecalis* strain DS16 (accession no. M85225). Together with the fact that these isolates indeed contained a member of the Tn*916*-Tn*1545* family (Table 3), the high sequence homology with reference sequences of Tn*916* seems to indicate that in these three isolates the *tet*(M) gene is integrated into a transposon of the latter type. It is noteworthy that the *tet*(M) gene of Tn*916*-like transposon Tn*FO1* (32) was closely linked to members of SHG III (9 to 13 nucleotide variations, mostly including unknown bases). The remaining two SHGs, IV and V, comprised the *tet*(M) genes of three Irish and two Italian cheese isolates of *E. faecalis*, respectively, and exhibited extremely high internal sequence homology (zero to one nucleotide substitution). Both SHGs did not include any other previously determined *tet*(M) sequences, and none of them were closely joined by known *tet*(M) sequences after similarity searches with the EMBL Fasta program, indicating that these sequences may represent two new phylogenetic lineages of *tet*(M). Like isolate LMG 20647, the two isolates of SHG V harbored a Tn*916*-Tn*1545* element but seemingly did not transfer any plasmids to JH2-2. Possibly, these *tet*(M)-containing isolates harbor Tn*916*-like structures previously found in *Enterococcus* spp. such as Tn*5381* (38), the *tet*(M) gene sequences of which are not available or even contain currently undescribed members of this conjugative-transposon family. Taken together, the main conclusion from our sequencing data is that transferable *tet*(M) genes carried by food enterococci belong to several different lineages, which may explain why this gene has been widely disseminated among at least 18 gram-positive and 8 gram-negative hosts (7).

Conclusions. Collectively, the data presented in this study demonstrate that Tc^r in food enterococci mainly originating from European cheeses is conferred basically by the same types of *tet* genes [*tet*(M), *tet*(L), and *tet*(S)] as those previously found among veterinary or clinical *Enterococcus* isolates. Moreover, the finding that a significant proportion of Tc^r isolates exhibited coresistance to ER and/or CM reinforces the suggestion that the selection of Tc^r genotypes may provide a suitable molecular basis for the further selection of multiple resistances (27). In vitro conjugation experiments showed that a considerable fraction of the *tet* genes in food enterococci is located on active mobile genetic elements including known and, potentially, undescribed members of the *tet*(M)-containing Tn*916*-Tn*1545* family. The high evolutionary diversity of *tet*(M) structures, as evidenced by the delineation of several SHGs, should provide the basis for further molecular studies. Next to physical linkage of *tet* and other resistance genes to enterococcal plasmids or chromosomal elements by Southern blotting, detailed molecular dissection of up- and downstream regions of the targeted *int* gene of Tn*916*/Tn*1545* and full sequence analysis of *tet* gene-containing self-transmissible plasmids need to be emphasized in follow-up studies. In order to establish the broader ecological and clinical relevance of Tc^r enterococci occurring along the food chain, extensive assessment of the in vitro and in vivo host range spectra of these *tet* gene carriers is needed. It is expected that these new insights will contribute to the definition of well-argued safety requirements for the approved use of *Enterococcus* strains as food starter cultures or as human or animal probiotics, an issue that is currently under debate in Europe (http://www.europa.eu.int /comm/food/fs/sc/scan/index_en.html) and the United States (http://www.fao.org/es/ESN/Probio/probio.htm).

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