Molecular Characterization of \textit{tet}(M) Genes in \textit{Lactobacillus} Isolates from Different Types of Fermented Dry Sausage

Dirk Gevers,\textsuperscript{1,*} Morten Danielsen,\textsuperscript{2} Geert Huys,\textsuperscript{1} and Jean Swings\textsuperscript{1,3}

Laboratory of Microbiology\textsuperscript{1} and BCCM/LMG Bacteria Collection,\textsuperscript{2} Faculty of Sciences, Ghent University, B-9000 Ghent, Belgium, and Identification Section, Applied Biotechnology, Chr. Hansen A/S, DK-2970 Hørsholm, Denmark\textsuperscript{2}

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The likelihood that products prepared from raw meat and milk may act as vehicles for antibiotic-resistant bacteria is currently of great concern in food safety issues. In this study, a collection of 94 tetracycline-resistant (Tc\textsuperscript{r}) lactic acid bacteria recovered from nine different fermented dry sausage types were subjected to a polyphasic molecular study with the aim of characterizing the host organisms and the \textit{tet} genes, conferring tetracycline resistance, that they carry. With the (GTG)\textsubscript{5}-PCR DNA fingerprinting technique, the Tc\textsuperscript{r} lactic acid bacterial isolates were identified as \textit{Lactobacillus plantarum}, \textit{L. sakei} subsp. \textit{carnosus}, \textit{L. sakei} subsp. \textit{sakei}, \textit{L. curvatus}, and \textit{L. alimentarius} and typed to the intraspecies level. For a selection of 24 Tc\textsuperscript{r} lactic acid bacterial isolates displaying unique (GTG)\textsubscript{5}-PCR fingerprints, \textit{tet} genes were determined by means of PCR, and only \textit{tet}(M) was detected. Restriction enzyme analysis with \textit{AccI} and \textit{ScaI} revealed two different \textit{tet}(M) allele types. This grouping was confirmed by partial sequencing of the \textit{tet}(M) open reading frame, which indicated that the two allele types displayed high sequence similarities (>99.6\%) with \textit{tet}(M) genes previously reported in \textit{Staphylococcus aureus} MRSA 101 and in \textit{Neisseria meningitidis}, respectively. Southern hybridization with plasmid profiles revealed that the isolates contained \textit{tet}(M)-carrying plasmids. In addition to the \textit{tet}(M) gene, one isolate also contained an \textit{erm}(B) gene on a different plasmid from the one encoding the tetracycline resistance. Furthermore, it was also shown by PCR that the \textit{tet}(M) genes were not located on transposons of the Tn\textsubscript{916}/Tn\textsubscript{1545} family. To our knowledge, this is the first detailed molecular study demonstrating that taxonomically and genotypically diverse \textit{Lactobacillus} strains from different types of fermented meat products can be a host for plasmid-borne \textit{tet} genes.

For several decades, studies on the selection and dissemination of antibiotic resistance have focused mainly on clinically relevant bacterial species. More recently, many investigators speculated that commensal bacteria may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens (30; S. B. Levy and A. A. Salyers, Reservoirs of Antibiotic Resistance [ROAR] Network, http://www.healthscinet.tufts.edu/apua/Roar/roarhome.htm, 2002). Such commensal reservoir bacteria can be present in the intestines of farmed animals exposed to antibiotics (2, 38) and may subsequently contaminate the raw meat produced from these animals even when hygiene regulations are respected. Several examples of antibiotic-resistant lactic acid bacteria isolated from raw meat exist (20, 26, 28, 37). Fermented foods prepared from raw meat or milk can therefore be considered potential vehicles for the spread of antibiotic-resistant lactic acid bacteria along the food chain to the consumer (35).

Genes conferring resistance to tetracycline, chloramphenicol, erythromycin, and vancomycin have been detected and characterized in \textit{Lactococcus lactis} (27) and enterococci (15, 35) isolated from fermented meat and milk products. In contrast, no molecular data are available on the occurrence of antibiotic resistance genes in lactobacilli present in fermented food products. Members of the genus \textit{Lactobacillus} also constitute an important part of the natural microflora associated with fermented products and are indigenous to the animal and human gastrointestinal tract. These properties make lactobacilli, along with enterococci, interesting indicator organisms with which to study the molecular ecology of antibiotic resistance determinants in food fermentation industries.

Previously, we isolated tetracycline-resistant (Tc\textsuperscript{r}) strains of various \textit{Lactobacillus} species from different types of modified atmosphere-packed fermented dry sausage (FDS) sold in Belgian retail shops (13). All \textit{Lactobacillus} isolates collected in this way were resistant to at least 64 \mu g of tetracycline per ml as determined with the de Man, Rogosa, and Sharpe (MRS) agar dilution method (13). The aim of the present investigation was to perform a molecular characterization of the \textit{tet} genes conferring the high-level phenotypic resistance to tetracycline in these isolates. For this purpose, an extended collection of Tc\textsuperscript{r} \textit{Lactobacillus} isolates was subjected to (GTG)\textsubscript{5}-PCR fingerprinting (14) to reveal their taxonomic identity and genotypic diversity. Based on these results, a selected subset of different strain types were characterized further to assess the identity, the heterogeneity, and the locations of their \textit{tet} genes.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial isolates.} In this study, a total of 94 Tc\textsuperscript{r} lactic acid bacterial isolates recovered from 14 batches of nine different FDS types were included. A type is defined here as a specific product variety distributed under a specific commercial brand. Each type is designated with a number (FDS-01, FDS-02, FDS-06, FDS-07, FDS-08, FDS-09, FDS-11, FDS-12, and FDS-14). A batch is defined as a group of sausages belonging to the same type that were produced at one time and is indicated with a letter from A to E.
The isolation of 52 Tc lactic acid bacterial isolates was reported before (13). This collection was extended with 42 new Tc lactic acid bacterial isolates recovered similarly from other batches of fermented dry sausage end products. Isolates were recovered on the basis of colony morphology rather than relative abundance in order to obtain a set of isolates with the highest diversity. All these fermented dry sausages were prepared with a starter culture. All isolates were stored in a bead storage system (Microbank system; Pro-LAB Diagnostics, Warrington, United Kingdom) at –80°C and grown on MRS agar (Difco Co., Detroit, Mich.) at 30°C under microaerophilic conditions (3.75% CO₂–5% O₂–7.5% H₂–83.75% N₂).

( GTG₅)₃-PC R fingerprinting. All Tc lactic acid bacterial isolates were identified at the species level and typed in the intraspecies level with high-resolution rep-PCR fingerprinting with the (GTG₅)₃ primer as previously described (14).

Antibiotic susceptibility testing and MIC determination. The PCR assay mix (total volume, 50 μl) contained 20 pmol of each primer (Table 1), 1× PCR buffer (Applied Biosystems, Warrington, United Kingdom), each deoxynucleotide triphosphate at a concentration of 200 μM, and 1 U of AmpliTaq DNA polymerase (Applied Biosystems, Warrington, United Kingdom). A 50-ng portion of purified total DNA was used as a template. In a first PCR assay, tet genes encoding ribosomal protection proteins (RPP) were detected with degenerate primers DI and DII (7). If positive for RPP genes, additional PCR assays were performed with primers specific for tet(M), tet(O), and tet(S) (Table 1). Next to the RPP tet genes, isolates were also tested for the presence of the tetracycline efflux genes tet(K) and tet(L) and for the transposon integrase gene (int gene) of the Tn1545/Tn1545 family (Table 1). One strain expressed erythromycin resistance was analyzed with erm(B)-specific primers as described previously (19).

All PCR amplifications were performed in a GeneAmp 9600 PCR system (Perkin-Elmer) with the following temperature program: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. PCR products (5 μl) were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

Sequencing of PCR products. For tet(M)-positive isolates, the purified PCR products obtained with the DI and Tet-M-R primers were directly sequenced with the primers DI, DII, and Tet-M-R (Table 1). Sequencing was performed with a BigDye Terminator version 2 Ready Reaction cycle sequencing kit (Applied Biosystems, Warrington, United Kingdom) on an ABI Prism 310 genetic analyzer (Applied Biosystems, Warrington, United Kingdom). On-line similarity searches were performed with the BLAST (Basic Local Alignment Search Tool) family of programs in GenBank.

Nucleotide sequence accession numbers. The sequences of the tet(M) genes described in this paper have been assigned GenBank accession numbersAY149574 to AY149597.

**RESULTS**

Identification of isolates. With (GTG₅)₃-PCR fingerprinting, the 94 Tc lactic acid bacterial isolates were identified as Lactobacillus plantarum (n = 31), L. sakei subsp. casei (n = 31), L. sakei subsp. salivarius (n = 9), L. sakei subsp. buchneri (n = 7), L. fermentum (n = 6), L. paracasei subsp. paracasei (n = 6), L. reuteri (n = 4), L. sakei subsp. sakei (n = 2), and L. brevis (n = 1).
46), L. sakei subsp. sakei (n = 5), L. curvatus (n = 7), and L. alimentarius (n = 5). Only one species was isolated from the majority of the positive samples (64%), whereas in all other cases no more than two species were identified. In some cases, different species compositions were obtained in different batches of the same sausage type, e.g., batches FDS-07A and -07B and FDS-08A and -08C. Numerical analysis of the (GTG)\(_2\)R-PCR fingerprints showed that isolates originating from the same batch often displayed identical banding patterns (results not shown). Based on pattern similarity, the set of isolates was reduced to one isolate per unique (GTG)\(_2\)R-PCR fingerprint type, which resulted in a collection of 24 Tc\(^{r}\) Lactobacillus isolates (Fig. 1).

**Phenotypic characterization of resistance.** Etest revealed that the MIC of tetracycline ranged between 32 and 256 \(\mu\)g/ml (upper limit of test), and the MIC for 50% of strains was \(>256\) \(\mu\)g/ml (Fig. 1). Next to the high-level tetracycline resistance found among the 24 selected isolates, some strains showed an additional phenotypic resistance to erythromycin (\(n = 1\)), rifampin (\(n = 4\)), and penicillin (\(n = 1\)) in disk diffusion susceptibility testing.

**Detection, characterization, and localization of resistance genes.** Total genomic DNA preparations from all 24 Tc\(^{r}\) Lactobacillus isolates were subjected to PCR amplification with the RFP set of primers, with class-specific primers for tet(M), tet(O), tet(S), tet(K), and tet(L) and with primers for detection of int genes of the Tn916/Tn1545 family of transposons. In all 24 isolates, only tet(M) was detected, and no int genes were found. Restriction enzyme analysis of the tet(M) PCR product (74% of the open reading frame) with AccI and ScaI revealed two different tet(M) allele types, tet(M)-I and tet(M)-2 (Fig. 1). Most batches contained strains belonging to one allele type, except for batches FDS-02-B, FDS-07-A, and FDS-14, which contained strains displaying both allele types (Fig. 1).

In order to characterize the tet(M) genes more profoundly, the open reading frame was partially (74%) sequenced from approximately position 280 (DI primer) to position 1700 (TetM-R primer). Sequence alignments revealed two sequence homology groups and one mosaic gene (Fig. 2). Between the homology groups, a difference of at least 25 bases was found. A first group comprised nine isolates in which the sequenced part of the tet(M) gene showed at most five base differences with the tet(M) gene of Neisseria meningitidis (GenBank accession no. X75073) (12). This group corresponds to tet(M)-I found by restriction enzyme analysis. A second group of 14 isolates with a maximum of three base differences with the tet(M) gene of Staphylococcus aureus MRSA 101 (GenBank accession no. M21136) (23) corresponded to tet(M)-2 defined by restriction enzyme analysis.

The tet(M) gene of isolate DG 13 exhibited a mosaic structure, combining partial sequences of the two foregoing homology groups. The sequence up to position 1508 of the open reading frame displayed one base difference with the tet(M) gene of Neisseria meningitidis and was identical to the tet(M) gene of Staphylococcus aureus MRSA 101 in the remaining part. Restriction enzyme analysis classified the tet(M) gene of this strain as a tet(M)-2. The one isolate expressing phenotypic erythromycin resistance (DG 507) was shown to contain the erm(B) gene by PCR and partial sequencing of the PCR product.

Plasmid profiling of the 24 Tc\(^{r}\) Lactobacillus isolates showed that 23 isolates (excluding DG 524) contained at least one and usually more than one plasmid (Fig. 1). By Southern blotting and hybridization with a tet(M)- and/or erm(B)-specific probe, the tet(M) genes of 20 isolates and the erm(B) gene in isolate DG 507 could be localized on a plasmid. Most of these R-plasmids had a size of approximately 10 kb, and in a few cases the R-plasmid was larger than 25 kb. Most plasmid profiles showed more than one band (up to three) that hybridized with the tet(M) probe (Fig. 1), due to hybridization with low concentrations of open circular and/or linear plasmid forms. For the remaining four Tc\(^{r}\) Lactobacillus isolates that did not carry the tet(M) gene on a plasmid (DG 142, DG 484, DG 524, and DG 525), Southern blots of EcoRI-digested DNA hybridized to the tet(M) probe, with fragments of between 8 and 12 kb (results not shown).

**DISCUSSION**

To our knowledge, this is the first detailed molecular study of antibiotic resistance genes in Lactobacillus species isolated from fermented dry sausages. The presence of antibiotic-resistant Lactobacillus species has been documented in wine, cheese (17, 34), poultry, calf, swine (11, 21, 33, 36), pig feces (4, 10, 29), healthy human feces (18), and maize silage (8). In the first phase of the current study, a total of 94 tetracycline-resistant lactic acid bacterial isolates recovered from 14 batches representing nine different fermented dry sausage types were identified by (GTG)\(_2\)R-PCR fingerprinting. All strains could be identified as Lactobacillus species commonly associated with fermented meat products, i.e., L. plantarum, L. sakei subsp. carnosus, L. sakei subsp. sakei, L. curvatus, and L. alimentarius (16).

In four of the batches investigated, isolates belonged to two different Lactobacillus species, and in five batches more than one (GTG)\(_2\)R-PCR fingerprint type per species was found (results not shown). Different batches of one fermented dry sausage type were not found to contain identical (GTG)\(_2\)R-PCR fingerprints, indicating that the source of Tc\(^{r}\) lactobacilli is variable. Interestingly, isolates DG 165, DG 485, and DG 516, originating from three different fermented dry sausage types (FDS-11B, FDS-09B, and FDS-06, respectively), displayed identical DNA fingerprints and harbored identical R-plasmids and tet(M) genes, suggesting a possible common source of Tc\(^{r}\) Lactobacillus contamination. In order not to select multiple isogenic strains, one strain each of the 24 unique (GTG)\(_2\)R-PCR fingerprint types was selected.

Given the fact that the Tc\(^{r}\) Lactobacillus isolates were recovered from five different fermented dry sausage types and contained 24 (GTG)\(_2\)R-PCR fingerprint types representing five different Lactobacillus species, it was somewhat surprising that in all isolates, of the five tet genes tested, only tet(M) was detected. According to current insights, tet(M) is the most widely distributed tet gene, being detected in at least eight gram-negative and 18 gram-positive genera, including the lactic acid bacterial genera Enterococcus, Streptococcus, and Bifidobacterium (6). It was suggested that the origin of tet(M) is most probably the tetracycline-producing species of Streptomyces and that its integration into mobile genetic elements (plas-
FIG. 1. Composite figure of (GTG)5-PCR fingerprints and inverted plasmid profiles of the 24 Tcr Lactobacillus isolates from fermented dry sausage products. The dendrogram was constructed after cluster analysis of the digitized (GTG)5-PCR fingerprints with the unweighted pair group method with arithmetic averages, with correlation levels expressed as a percentage of the Pearson correlation coefficient. Cophenetic correlations (shown on each branch of the dendrogram) indicate how faithfully the dendrogram represents the similarity matrix. A small triangle or circle indicates the place where the tet(M) or tet(B) probe, respectively, hybridized on the Southern blot of the plasmid DNA. Strain Lactococcus lactis subsp. cremoris strain AC1 was used as a plasmid size marker (24). The tet(M)-1 and tet(M)-2 allele types correspond to the tet(M) genes found in Neisseria meningitidis (X75073) and Staphylococcus aureus MRSA 101 (M21136), respectively, based on restriction enzyme analysis (REA) with AccI and ScaI. Numerical designations indicate the fermented dry sausage type, and the letters A to D indicate different batches (type and batch are defined in Materials and Methods). tet(M) localization was determined by Southern blot analysis. The MIC of tetracycline was determined by Etest. Chr., chromosomal band.
mosome ($n = 4$) or on a plasmid ($n = 10$). R-plasmids encoding tetracycline, chloramphenicol, gentamicin, and macrolide-lincosamide-streptogramin B resistance have been reported previously in *L. reuteri* (4, 21, 33, 36), *L. fermentum* (10, 18), *L. acidophilus* (36), and *L. plantarum* (1, 8) isolated from raw meat, feces, and maize silage. Most of these R-plasmids were smaller than 10 kb. The tetracycline MIC was significantly lower for the Tc' *Lactobacillus* isolates with a chromosomal tet gene in this study than for those with plasmid-encoded tetracycline resistance, 32 to 48 μg/ml and >192 μg/ml, respectively, with the exception of two *L. sakei* subsp. *carnosus* isolates (DG 488 and DG 489). More research is needed to investigate to what extent this marked difference in phenotypic resistance levels is linked to, e.g., the location of the tet(M) gene or the copy number of the R-plasmid.

In conclusion, the results of the current study indicate that *Lactobacillus* species from fermented meat products can harbor acquired tetracycline resistance encoded by a tet(M) gene, which is usually located on a plasmid and displays very high genotypic similarities with tet(M) genes previously reported in two pathogenic species. Further research may focus on the diversity and transferability of these *Lactobacillus* plasmids into other commensal bacteria and on the source of Tc' lactobacilli in the production of fermented dry sausages.

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