Applied and Environmental Microbiology, June 2009, p. 3866–3871 Vol. 75, No. 12
0099-2240/09/$08.00+0 doi:10.1128/AEM.00589-09

Characterization of the Tn*916* Conjugative Transposon in a Food-Borne Strain of *Lactobacillus paracasei*[▽]

Chiara Devirgiliis,[1,2] Doriana Coppola,[1] Simona Barile,[1] Bianca Colonna,[2] and Giuditta Perozzi[1]*

INRAN (National Research Institute on Food and Nutrition), Via Ardeatina 546, 00178 Rome, Italy,[1] and Department of Cell and Developmental Biology, Sapienza University of Rome, Via dei Sardi 70, 00185 Rome, Italy[2]

Received 11 March 2009/Accepted 16 April 2009

Food-borne antibiotic-resistant lactic acid bacteria have received growing attention in the past few years. We have recently identified tetracycline-resistant *Lactobacillus paracasei* in samples of milk and natural whey starter cultures employed in the manufacturing process of a typical Italian fermented dairy product, Mozzarella di Bufala Campana. In the present study, we have characterized at the molecular level the genetic context of tetracycline resistance determinants in these natural strains, which we have identified as *tet*(M). This gene was present in 21 independent isolates, whose fingerprinting profiles were distributed into eight different repetitive extragenic palindromic groups by cluster analysis. We provide evidence that the gene is associated with the broad-host, conjugative transposon Tn*916*, which had never before been described to occur in *L. paracasei*. PCR analysis of four independent isolates by use of specifically designed primer pairs detected the presence of a circular intermediate form of the transposon, carrying a coupling sequence (GGCAAA) located between the two termini of Tn*916*. This novel coupling sequence conferred low conjugation frequency in mating experiments with the recipient strain JH2-2 of *Enterococcus faecalis*.

Several genetic determinants conferring tetracycline resistance have been described to occur in gram-positive, nonpathogenic bacteria (2, 20). Among them, *tet*(M), encoding a ribosomal protection protein, is most commonly found in lactic acid bacteria (LAB). The issue of antibiotic resistance spreading among commensal bacteria has received great interest in recent years, and the presence of antibiotic-resistant species in the environment, including food products, has been extensively reported (reviewed in references 2 and 20). Conjugative transposons represent important vehicles for dissemination of antimicrobial resistance within gram-positive and gram-negative bacteria (23). These elements can move from the genome of a donor bacterium to that of a recipient by conjugation (6). Tn*916*, an 18-kb element containing the genetic determinant for tetracycline resistance, was the first conjugative transposon to be identified. It carries the *tet*(M) gene and has a broad host range, comprising both gram-positive and gram-negative bacteria (7). Along with the tetracycline resistance gene, Tn*916* carries the genes responsible for its own excision (*xis*) and integration (*int*) as well as the *mob* genes, which mediate conjugal transfer (4). The transposition process starts with excision of the transposon, mediated by the Int and Xis proteins, leading to the formation of a nonreplicative circular intermediate which is transferred to the recipient and integrates into a new target site. Excision represents the rate-limiting step and occurs through reciprocal, site-specific recombination between the nonhomologous regions located at the two termini of the integrated transposon, known as coupling sequences, which are retained in the circular intermediate (17).

Lactobacillus paracasei belongs to the microbial group of LAB and represents, along with the closely related species *Lactobacillus casei*, one of the most common bacterial species employed in the food industry. It is naturally present in raw milk and in dairy products, such as typical cheeses obtained by traditional manufacturing procedures in different Mediterranean countries (1, 11, 18, 26). Moreover, due to its probiotic functions, it is also employed as food additive (3, 5). Among its beneficial properties for human health, a recent study suggested that *L. paracasei* can be considered a potential enhancer of systemic immunity (22). However, only a few studies analyzed antibiotic resistance in *L. paracasei* (15, 19).

In the past few years, our studies have focused on the identification of genes responsible for antibiotic resistance in LAB isolated from traditional dairy foods manufactured without employing commercial starter cultures. Fermentation in such products is therefore carried out by natural starters, mostly reflecting the microbiological composition of raw milk, which is affected in turn by the environment in which the animals live. Moreover, selective pressure exerted by technological steps along the manufacturing procedure often has a deep impact on bacterial composition in the final product. The widespread use and misuse of antibiotics have applied strong selective pressure in the environment, favoring survival and spread of antibiotic-resistant species. It is therefore of special relevance to identify antibiotic resistance determinants in food-borne bacteria, their persistence along the production line of specific products, and their capability of horizontal transfer to those species that can colonize the human gut.

In the present study, we have characterized at the molecular level a group of tetracycline-resistant *L. paracasei* isolates, previously identified in raw milk and natural whey starter cultures employed in the manufacture of the Italian traditional cheese Mozzarella di Bufala Campana (9). We provide evidence that in these isolates, tetracycline resistance is due to the presence of the conjugative transposon Tn*916*, carrying the *tet*(M) gene

* Corresponding author. Mailing address: INRAN, Via Ardeatina 546, 00178 Rome, Italy. Phone: 39 06 51494 552. Fax: 39 06 51494 550. E-mail: perozzi@inran.it.

▽ Published ahead of print on 24 April 2009.

TABLE 1. Primers used in PCR amplifications

Primer pair	Name	Sequence	Annealing temp (°C)	Amplicon size (bp)	Source or reference
I	tetM-1 tetM-2	GAACTCGAACAAGAGGAAAGC ATG GAAGCCCAGAAAGGAT	60	740	21
II	Int-FW Int-RV	GCGTGATTGTATCTCACT GACGCTCCTGTTGCTTCT	50	1,028	12
III	Tet-int-FW Tet-int-RV	CGGATAGATAAAGTACGATA TCACGTCTTTTTTCTGACAT	52	2,659	This study
IV	For-ext Rev-ext	CATTCACATCGAAGTGCCGCCAAATCC GCTTTCCTCTTGTTCGAGTTCCAATGC	65	17,354	This study
V	nt 18K-FW Rev-ext	GCA AATGCAGGAATGAATCCAAAGG GCTTTCCTCTTGTTCGAGTTCCAATGC	60	13,042	This study
VI	int-tag-FW nt288-RV	AGAAGCAACAGGAGCGTC GCTGAATGAATGTTTGATGG	52	473	This study

and capable of horizontal, interspecies transfer to the opportunistic pathogen *Enterococcus faecalis* via a circular intermediate containing a novel coupling sequence that confers a low-frequency-conjugation phenotype. Molecular analysis of the resulting primary *E. faecalis* transconjugants revealed the presence of a circular intermediate of Tn*916* carrying the same coupling sequence found in the *L. paracasei* donor strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The major features of the *L. paracasei* strains used in this study are described in Fig. 2. These strains were routinely grown in MRS (Oxoid, Italy), with or without addition of tetracycline (Sigma, Italy), at 30°C for 24 to 48 h under anaerobic conditions (Anaerocult A; Merck, Germany). The strains used in mating experiments were *E. faecalis* JH2-2 (LMG 19456), resistant to rifampin (rifampicin) and fusidic acid, grown in brain heart broth (Merck, Germany) at 37°C; *Leuconostoc mesenteroides* M7-1 (LMG 19463), resistant to vancomycin, grown in MRS at 30°C, obtained from the BCCM/LMG Bacteria Collection, Belgium; and *E. faecalis* RE25 (25), kindly provided by Giorgio Giraffa, Lodi, Italy.

Filter matings. In vitro conjugation experiments were performed according to the method of Huys et al. (14). Donor and recipient strains were grown overnight in the appropriate selective media and diluted 1:5 in nonselective medium to late exponential growth phase. Strains at different donor/recipient ratios (1:1 and 10:1) were mixed and filtered through a 0.45-μm-pore-size membrane (HAWP 2500; Millipore, Italy) in a Swinnex filter holder (SX00 02500; Millipore). Filters were washed with 2 ml of peptone-physiological saline (NaCl [8.5 g/liter], peptone [1 g/liter]) and incubated on solid medium for 24 h under the optimal recipient growth conditions. Cells were detached from filters in 1 ml peptone-physiological saline, and serial dilutions were plated on brain heart agar plates supplemented with tetracycline (10 mg/liter), rifampin (50 mg/liter), and fusidic acid (100 mg/liter) or on MRS agar containing tetracycline, rifampin, and vancomycin (64 mg/liter). Both donor and recipient strains were unable to grow on triple selection. Transconjugant colonies were recovered following incubation at 37°C for 24 to 72 h and characterized by repetitive extragenic palindromic PCR (REP-PCR) fingerprinting and PCR-based detection of the *tet*(M) and *int* genes. Conjugation frequency is expressed as the ratio between the numbers of transconjugants/donor colonies.

DNA extraction and molecular analysis. Total DNA was obtained by microLYSIS (Microzone, Canada) or an FTA starter pack (Whatman, United Kingdom) kits, according to the manufacturer's instructions. Genomic DNA was extracted using a MagPrep bacterial genomic DNA kit (Merck, Germany). PCR mixtures contained 200 μM of each deoxynucleoside triphosphate, 1 μM of each forward and reverse primer (Table 1 and Fig. 1), 2 mM $MgCl_2$, and 2 U *Taq* DNA polymerase (Polymed, Italy) in the supplied buffer. An LA AccuTaq kit (Sigma) was used for amplification of fragments of >5 kb. PCR products were eluted from gels, purified with a NucleoSpin Extract II purification kit (Macherey-Nagel, Italy), and sequenced with the forward primer (M-Medical sequencing service, Italy). Southern hybridization was performed by standard protocols, using probes labeled with digoxigenin-11-dUTP (Roche Diagnostics, Italy). Restriction endonucleases were purchased from Promega (Italy).

REP-PCR fingerprinting. REP-PCR was performed with the $(GTG)_5$ primer, as described by Gevers et al. (13). The resulting profiles were analyzed with the Bionumerics version 4.5 software package (Applied Maths, Belgium). Similarity among digitized profiles was calculated using the Pearson correlation coefficient, and dendrograms were constructed with the unweighted-pair group method with arithmetic averages. The reproducibility of $(GTG)_5$ fingerprints was assessed by comparing PCR products obtained from three separate cultures of the same strain. The patterns for the same strain were about 90% similar (minimum level of reproducibility); consequently, profiles with ≥90% similarity were considered a single REP group.

RESULTS

***L. paracasei* harbors the *tet*(M) gene associated with Tn*916*.** Identification of 21 *L. paracasei* isolates displaying phenotypic resistance to tetracycline (MIC = 32 mg/liter) from an Italian cheese (Mozzarella di Bufala Campana DOP) was previously

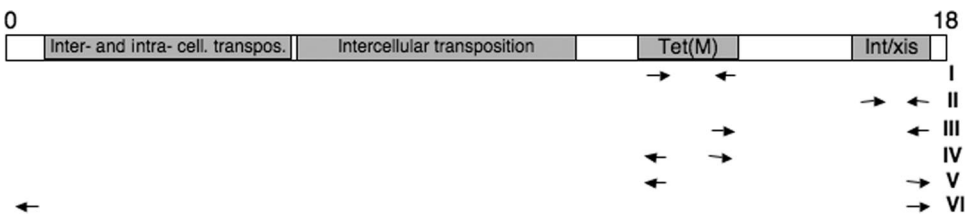

FIG. 1. Graphical representation of primer pairs along the Tn*916* genetic structure. Arrows represent the orientation of each primer and the relative positions of the primers along the linear sequence of the transposon. Primer pairs are indicated by Roman numerals.

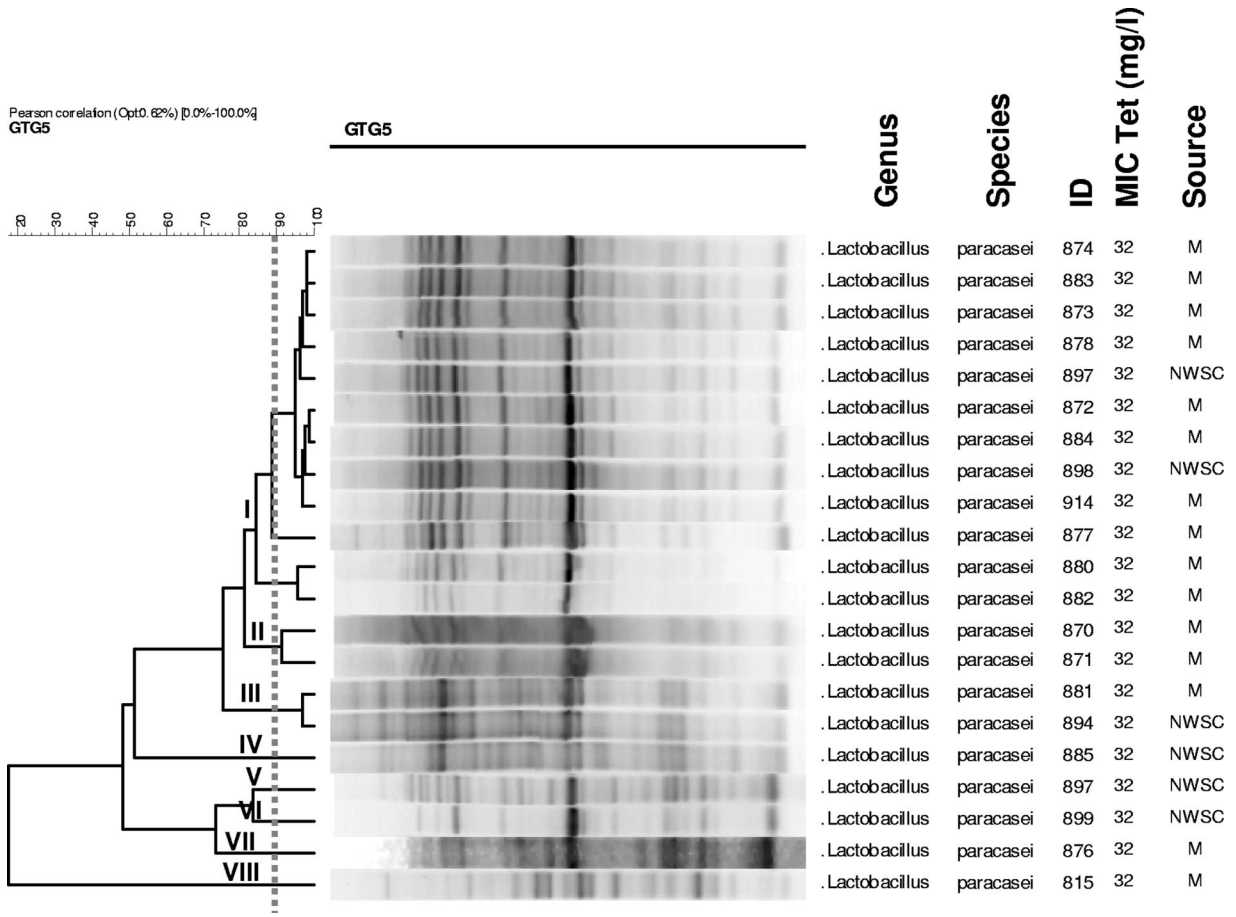

FIG. 2. Cluster analysis of $(GTG)_5$ PCR fingerprints of the tetracycline-resistant *L. paracasei* isolates. Clustering (expressed as percent similarity) was determined by the unweighted-pair group method with arithmetic averages and the Pearson product moment correlation coefficient. The vertical dotted line indicates 90% correlation (minimum level of reproducibility). Roman numbers indicate REP groups. Identification numbers for each independent isolate (ID), tetracycline MICs, and sources of isolation are indicated on the right. M, water buffalo raw milk; NWSC, natural whey starter cultures.

described (9). The isolates were found in raw milk and natural whey starter cultures from one of the three cheese factories analyzed. Figure 2 shows cluster analysis of the fingerprinting profiles of the isolates, obtained by REP-PCR, which identified eight different REP groups. Subsequent experiments were conducted either on all isolates or on representatives from each REP group. The presence of genes conferring tetracycline resistance was detected by PCR amplification of the most common *tet* genes found in lactobacilli. As shown in Fig. 3A (lane 2), amplification of an internal fragment of the *tet*(M) gene with primers tetM-1 and tetM-2 (Table 1) resulted in an amplicon of about 750 bp. This result was obtained for all isolates, irrespective of the REP group. However, primer pairs specific for the *tet*(S), *tet*(W), or *tet*(L) gene did not yield any amplicons (data not shown). Determination of the DNA sequence of the amplicons revealed a 100% match with the sequence of the Tn*916*-associated *tet*(M) gene (GenBank accession no. U09422). We therefore used a PCR strategy to verify whether the entire transposon was present in the genome of the tetracycline-resistant *L. paracasei* isolates. To this aim, some of the transposon-specific genes were amplified with the primers listed in Table 1 and represented in graphic format in Fig. 1, in which the position of each primer pair along the linear sequence of the transposon is shown. The results of this approach demonstrated the presence of the *int* gene, encoding the integrase protein that allows mobilization of Tn*916* (Fig. 3A, lane 3). Moreover, with the use of primers designed on the basis of the *tet*(M) and *int* sequences to amplify the intervening region between the two genes, the expected 2.6-kb fragment was detected (Fig. 3A, lane 4), demonstrating genetic linkage. Southern blot analysis with a *tet*(M) probe showed a single EcoRI band of about 20 kb, indicating that only one integration event occurred within the *L. paracasei* chromosome (Fig. 3B).

Tn*916* forms a circular intermediate in *L. paracasei*. The Tn*916* transposon was shown to transfer between different genomes via a nonreplicative circular intermediate (7). To investigate whether such mechanism occurred also in *L. paracasei*, we designed two primers directed outwards from the *tet*(M) gene which could yield an amplicon only in the case of Tn*916* circularization (primer pair IV) (Fig. 1). The results of PCR amplification of total DNA extracted from one isolate (Fig. 2, independent isolate 883) revealed the presence of a band of about 17 kb (Fig. 3C, lane 4), corresponding to the expected size of the full-length transposon lacking the *tet*(M)

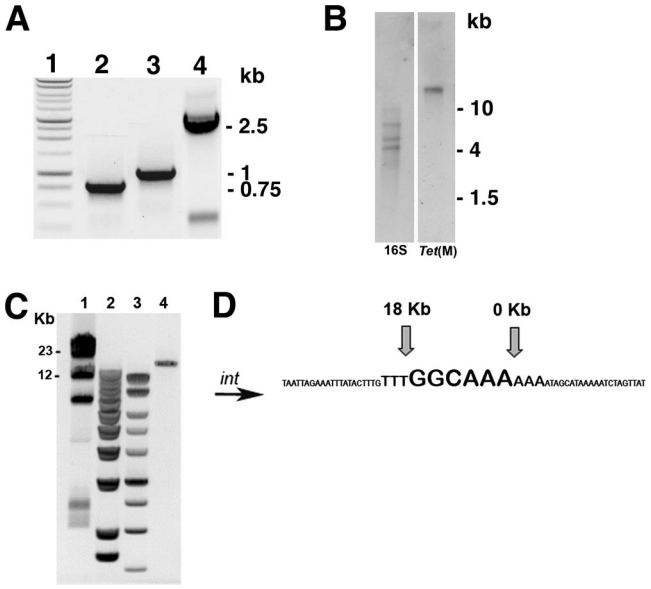

FIG. 3. *tet*(M) is associated with Tn*916* and integrates as a single copy through a circular intermediate form. (A) PCR amplification of Tn*916* regions. Lane 1, 1-kb DNA ladder ranging between 250 bp and 10 kb; lane 2, *tet*(M) (primer pair I); lane 3, *int* (primer pair II); lane 4, *tet*(M)-*int* (primer pair III). The numbering of the primer pairs and their positions along Tn*916* are given in Table 1 and Fig. 1. (B) Southern blot analysis of EcoRI-digested genomic DNA, probed with 16S (left panel) and *tet*(M) (right panel) gene fragments. (C) Lane 1, HindIII-digested lambda phage DNA size marker; lane 2, 1-kb DNA ladder plus DNA marker (100 bp to 12 kb); lane 3, 1-kb DNA ladder (250 bp to 10 kb); lane 4, PCR amplification of total DNA with primer pair IV (Table 1). (D) DNA sequence of the joined termini of the *L. paracasei* Tn*916* circular intermediate, obtained using the amplicon in panel C, lane 4, as a template. The coupling sequence (GGCAAA) and the interrupted DraI restriction site (TTTAAA) are indicated with larger lettering. Arrows indicate the initial and terminal nucleotides of the linear Tn*916* sequence (GenBank accession no. U09422).

intervening sequence between the two primers. These results conclusively demonstrate the existence of a circular intermediate of Tn*916* in *L. paracasei* independent isolate 883 and were confirmed by amplification of DNA extracted from three other independent isolates (Fig. 2, independent isolates 872, 882, and 898). The same result was obtained with the use of different primer pairs (V and VI) (Table 1) designed on the basis of Tn*916* regions encompassing other genes and resulting in different amplicon sizes (Fig. 4A, lane 1, and data not shown). Partial sequencing of the amplicons across the region of the joined termini identified a 6-bp insertion (GGCAAA) within the canonical sequence of Tn*916* (Fig. 3D). Six extra nucleotides flanking the insertion point of Tn*916* were previously described to occur in other species and named coupling sequences (7). Insertion of such sequence was postulated to occur during excision of the transposon, which represents the first step of circularization (6). In our isolates, the coupling sequence maps, as expected, between the two termini of the transposon (nucleotides 1 and 18032), and its insertion leads to destruction of a putative DraI site (Fig. 3D), which would be present only in the circular form of Tn*916*, as it is created after joining of the two linear termini. The DraI and MspI restriction patterns of the circular intermediate obtained with primer pair V lacking the region between *tet*(M) and *int* (Fig. 4A) were compared with the corresponding theoretical maps of Tn*916* (GenBank accession no. U09422) obtained with NEB cutter version 2.0 software (27) (Fig. 4B and C). The numbers and the lengths of the resulting restriction fragments confirmed the presence of all those expected when the DraI site encompassing the site of circularization is disrupted (Fig. 4). Moreover, the presence of the full-length Tn*916* transposon in our *L. paracasei* isolates is demonstrated by the two amplicons obtained with primer pairs III and V (Fig. 3A and 5A) as well as by partial sequencing of some of the amplicons.

Tn*916* is horizontally transferred to *E. faecalis* JH2-2 at a low frequency. Since Tn*916* is a conjugative transposon in *Enterococcus*, we asked whether it could be horizontally trans-

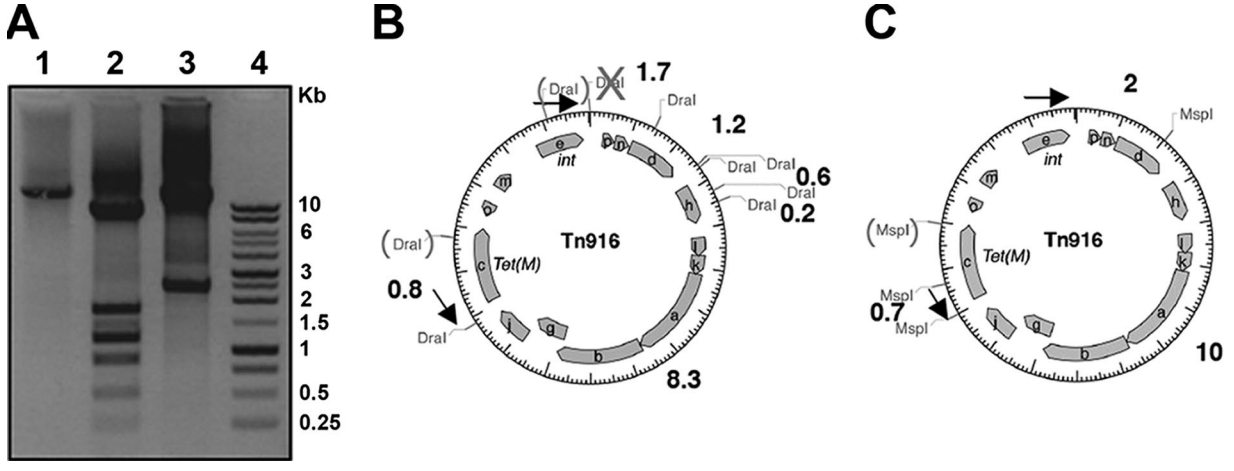

FIG. 4. Restriction analysis of the *L. paracasei* Tn*916* amplicon containing the coupling sequence. (A) Agarose gel electrophoresis of the amplicon obtained with primer pair V (Table 1). Lane 1, undigested control; lane 2, DraI digestion; lane 3, MspI digestion; lane 4, size marker (250 bp to 10 kb). (B and C) Theoretical restriction maps, obtained with NEB cutter version 2.0, of the circular form of Tn*916* (GenBank accession no. U09422), indicating the positions of the DraI (B) and MspI (C) restriction sites. Arrows indicate primers used in PCR amplifications. Restriction sites mapping outside the amplified fragment are shown in parentheses. The DraI site interrupted by the coupling sequence is crossed. Fragment sizes (kb) are indicated between restriction sites.

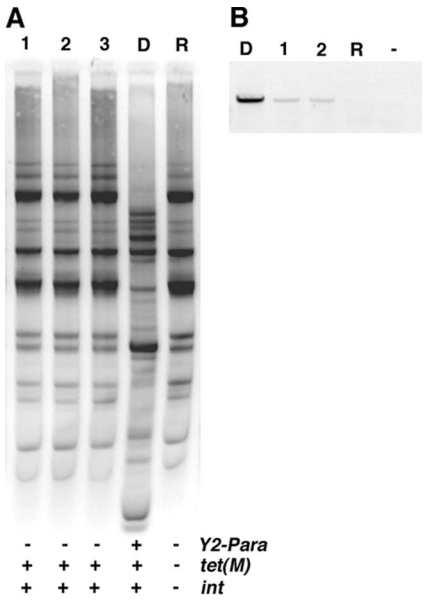

FIG. 5. Analysis of *E. faecalis* JH2-2 transconjugants. (A) $(GTG)_5$ PCR fingerprinting patterns of independent *E. faecalis* transconjugants (lanes 1 to 3), the *L. paracasei* donor strain (D), and the *E. faecalis* JH2-2 recipient strain (R). (B) PCR amplification of the Tn*916* circular intermediate with primer pair IV (Table 1). Results are shown for the *L. paracasei* donor strain (D), the independent transconjugants (lanes 1 and 2), the *E. faecalis* JH2-2 recipient strain (R), and the negative control lacking template DNA (−).

ferred from *L. paracasei* donors to the recipient strain *E. faecalis* JH2-2 in interspecies mating experiments. However, the results indicated a low conjugal transfer frequency (1×10^{-8}), with recovery of few transconjugant colonies only when a high donor/recipient ratio (10:1) was used. Molecular analysis of the phenotypically tetracycline-resistant transconjugants showed that they harbored both the *tet*(M) and the *int* genes while displaying the fingerprinting profile of the recipient strain (Fig. 5A), which confirms transfer of the Tn*916* transposon bearing *tet*(M). The same transconjugants were also analyzed for the presence of a circular Tn*916* intermediate by amplification of total DNA with primer pair IV (Table 1). As shown in Fig. 5B, we detected the presence of an amplicon of the expected molecular size (about 17 kb), even though this amplicon was less intense than that in the *L. paracasei* donor strain. The same result was obtained with the use of a different primer pair (VI) (Table 1) that amplifies a smaller fragment of about 500 bp contained within the primer pair V amplicon and encompassing the region of circularization (data not shown). Sequencing of the latter amplicon revealed the presence of the same insertion sequence found in *L. paracasei* (GGCAAA).

Primary transconjugants as donors of Tn*916*. The coupling sequence was shown to affect conjugation frequency in *E. faecalis* (17). Since the coupling sequence of the Tn*916* element in our tetracycline-resistant *L. paracasei* isolates was different from all those previously described, we tested the ability of primary *E. faecalis* transconjugants to act as Tn*916* donors in subsequent mating experiments. In these experiments, we used the vancomycin-resistant *L. mesenteroides* strain M7-1 as a recipient, because the transconjugant donors carried both tetracycline and rifampin resistance determinants from the original donor and recipient strains. Three independent filter mating experiments did not yield any transconjugants on plates containing rifampin, tetracycline, and vancomycin, while the positive control donor strain *E. faecalis* RE25 yielded several transconjugant colonies (data not shown).

DISCUSSION

Tetracycline resistance in *L. paracasei* is still poorly understood. To the best of our knowledge, only one recent report describes the presence of the *tet*(M) gene in isolates belonging to this species (15). In this study, we present a detailed investigation of the *tet*(M) genetic context in eight tetracycline-resistant REP groups of *L. paracasei*, isolated from raw water buffalo milk and natural whey starter cultures sampled at a processing line for Mozzarella di Bufala Campana, a traditional PDO (Protected Designation of Origin) fermented cheese typical of Southern Italian regions. Using a PCR strategy with specifically designed oligonucleotide primers, we have shown in these isolates the presence of the *tet*(M) gene within the Tn*916* transposon, a conjugative element originally identified in a strain of *E. faecalis* and capable of mediating intercellular transposition primarily among gram-positive bacteria (6). Our work demonstrates for the first time the presence of such conjugative element in *L. paracasei*.

It is known that the excision-insertion process necessary for intercellular transfer of Tn*916* starts with the formation of a nonreplicative circular intermediate (6, 24). PCR amplifications with primers encompassing the terminal sequences of the transposon revealed the presence in *L. paracasei* of a circular form, containing an insertion sequence of 6 nucleotides between the two joined termini of the linear Tn*916* sequence. Such sequences have previously been described to occur in other species and named coupling sequences. They result from reciprocal, site-specific, nonhomologous recombination at the excision site, and their specific sequence was found to determine the transfer efficiency of the transposon (reviewed in reference 6). Among the characterized sequences, TTAGTT, ATTTTA, TAAACT, and ATGGAA result in a high-conjugation-frequency phenotype, while AATCAA and GTTAAA lead to low conjugation frequency (17). The coupling sequence described in this study is novel (GGCAAA), and our results indicate that it confers a low-conjugation-frequency phenotype, as we recovered very few transconjugants when mating *L. paracasei* donors with the *E. faecalis* JH2-2 recipient and no transconjugants at all when mating the resulting *E. faecalis* Tn*916*-bearing transconjugants with an *L. mesenteroides* recipient. Moreover, molecular characterization of the *E. faecalis* transconjugants had shown the presence of a Tn*916* circular intermediate containing the same coupling sequence as the donors. The low transfer frequency of the tetracycline resistance determinant identified in our food-borne strains of *L. paracasei* to the opportunistic pathogen *E. faecalis* is especially relevant, as these two species coexist in the crowded environment of the human gastrointestinal tract. Recent studies report a wide spectrum of frequencies of transfer of the *tet*(M) gene from *Lactobacillus* and *Lactococcus* species to *E. faecalis* (8, 10, 16), suggesting that the genetic background strongly affects conjugation efficiencies. Moreover, the frequency of transconjugants recovered from in vitro filter mating experiments differs

from that obtained with in vivo procedures (16). We cannot, therefore, rule out the possibility that the transfer frequency of the *tet*(M)-bearing Tn*916* transposon described in this study might differ under natural conditions, such as the gastrointestinal tract or dairy environment.

Taken together, our results have contributed to a deeper understanding of the spreading and evolution of antibiotic resistance genes within environmental microflora. Moreover, the low conjugation frequency of tetracycline-resistant *L. paracasei* carrying the *tet*(M) gene in the conjugative transposon Tn*916* points at a reduced risk of horizontal transfer to pathogenic species within the human gut microflora.

ACKNOWLEDGMENTS

We thank Kariklia Pascucci for her kind support in daily laboratory work.

This work was supported by grant ARAFOA (DM 662/7303/2003) from the Italian Ministry of Agriculture, Food & Fisheries (MiPAAF).

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