Transfer of Antibiotic Resistance Marker Genes between Lactic Acid Bacteria in Model Rumen and Plant Environments

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Three wild-type dairy isolates of lactic acid bacteria (LAB) and one Lactococcus lactis control strain were analyzed for their ability to transfer antibiotic resistance determinants (plasmid or transposon located) to two LAB recipients using both in vitro methods and in vivo models. In vitro transfer experiments were carried out with the donors and recipients using the filter mating method. In vivo mating examined transfer in two natural environments, a rumen model and an alfalfa sprout model. All transconjugants were confirmed by Etest, PCR, pulsed-field gel electrophoresis, and Southern blotting. The in vitro filter mating method demonstrated high transfer frequencies between all LAB pairs, ranging from 1.8 × 10⁻⁵ to 2.2 × 10⁻² transconjugants per recipient. Transconjugants were detected in the rumen model for all mating pairs tested; however, the frequencies of transfer were low and inconsistent over 48 h (ranging from 1.0 × 10⁻⁹ to 8.0 × 10⁻⁶ transconjugants per recipient). The plant model provided an environment that appeared to promote comparatively higher transfer frequencies between all LAB pairs tested over the 9-day period (transfer frequencies ranged from 4.7 × 10⁻⁴ to 3.9 × 10⁻¹ transconjugants per recipient). In our test models, dairy cultures of LAB can act as a source of mobile genetic elements encoding antibiotic resistance that can spread to other LAB. This observation could have food safety and public health implications.

Lactic acid bacteria (LAB) form a taxonomically diverse group of gram-positive, catalase-negative microorganisms, which share the capacity to ferment sugars into lactic acid. Due to their aerotolerant, anaerobic nature, they are found widespread in a variety of different environments. Traditionally LAB are economically important given their use in the manufacture and preservation of fermented foods, such as milk, meat, vegetables, and cereals, in addition to their use as starter cultures. Over the last 2 decades, there has been an increased focus on the health-promoting properties associated with increased ingestion of probiotic LAB. As a result of these health claims, there is an increased availability of commercially prepared probiotic products, including yogurts, milk, cheeses, and even probiotic supplements in tablet form.

The global spread of antibiotic resistance, including the emergence of multiresistant bacterial “super bug” strains, has created a public health problem of potentially crisis proportions. The very success of antibiotics accounts for part of the reason for the problem; overuse of antibiotic treatments in both humans and animals has selected for a rapid increase of resistant bacterial strains. Acquired resistance genes may transfer by conjugation, transformation, or transduction. However, with regard to horizontal gene transfer (HGT), conjugation (which involves the use of plasmids or conjugative transposons as vehicles for resistance determinants) is thought to have the most significant impact on the spread of resistance genes in the environment (5).

Genes conferring acquired resistance to antibiotics such as tetracycline, erythromycin, and vancomycin have been detected in LAB isolated from fermented meat and milk products (3, 6, 8, 9, 11, 22, 37). Conjugal plasmids and transposons are common in LAB (1, 4), and due to their wide environmental distribution, it is possible that these commensal bacteria act as vectors for the dissemination of antibiotic resistance determinants to the consumer via the food chain (8, 24, 32). Such evidence has raised questions regarding LAB’s traditionally accepted safety status and initiated investigations in the biosafety of probiotic products (35). However, no consensus for testing the safety of LAB probiotic products exists at the European level.

To date, most of the research assessing the risk posed by the dissemination of resistance genes by LAB has been laboratory-based studies using in vitro mating models. Knowledge concerning HGT in the natural environment is limited (23, 39), and evidence is often circumstantial and extrapolated from laboratory-based studies (4). In order to fully understand the extent to which LAB strains transfer resistance genes in the natural environment, it is essential to study genetic exchange in this context. The rumen may be considered a site for potential conjugal gene transfer due to the following features: (i) its high bacterial density (10¹⁰ cells ml⁻¹); (ii) available surfaces suitable for the attachment of bacteria, including substrate particles and the rumen wall; and (iii) frequent seeding of the rumen with soil and plant microorganisms. Similarly, alfalfa sprouts provide a suitable plant model to investigate in vivo conjugal transfer between LAB strains due to their basic growth requirements (for instance, no soil is involved in growing, so therefore, background flora is eliminated), and natural LAB strains are known to colonize sprouts, so there is a good chance of survival once inoculated (16).
Filter mating. The aim of this study was to examine the horizontal transfer of tetracycline and erythromycin resistance determinants from three wild-type LAB strains, using both an in vitro mating method and in vivo models. Impacts of this transfer are discussed in the light of food safety and potential effects on public health.

MATERIALS AND METHODS

Bacterial strains. Four LAB strains were used as donors and two LAB strains were used as recipients in mating experiments. All strains used in this study are listed in Table 1. The Lactococcus lactis strains were grown in M17 broth medium (CM0017; Oxoid, Basingstoke, Hampshire, United Kingdom), in which lactose was replaced by 1% (wt/vol) glucose (G752; Sigma-Aldrich, St. Louis, MO) and incubated at 30°C. Streptococcus thermophilus was grown in M17 broth medium and incubated at 37°C. Enterococcus faecalis was grown in brain heart infusion (BHI) medium (CM0225; Oxoid) and incubated at 37°C. All strains were preserved in a bead storage system (Protect bacterial preservers; TSC Ltd., Heywood, United Kingdom) at −20°C and were routinely recultured.

Antibiotic (Sigma-Aldrich) concentrations used for selective plating were as follows; for donor enumeration, GM17 agar containing 5 µg ml⁻¹ erythromycin (L. lactis SH4174), GM17 agar containing 10 µg ml⁻¹ tetracycline (L. lactis 477 and 487), and M17 agar containing 256 µg ml⁻¹ erythromycin (S. thermophilus E2); for recipient enumeration, GM17 agar containing 100 µg ml⁻¹ rifampin and 100 µg ml⁻¹ streptomycin (L. lactis BU-260) and BHI agar containing 50 µg ml⁻¹ rifampin and 25 µg ml⁻¹ fusidic acid (E. faecalis HJ2-2); and for transconjugant enumeration, GM17 agar containing 5 µg ml⁻¹ erythromycin or 10 µg ml⁻¹ tetracycline, 100 µg ml⁻¹ rifampin, and 100 µg ml⁻¹ streptomycin and BHI agar containing 256 µg ml⁻¹ erythromycin, 50 µg ml⁻¹ rifampin, and 25 µg ml⁻¹ fusidic acid.

Inoculum preparation. Prior to the mating experiments, strains were plated for single colonies from a stock at −20°C. After overnight incubation, one to two colonies were picked and grown in 20 ml broth. Donors were grown in broth media supplemented with antibiotics (Sigma-Aldrich), with concentrations as described above. Recipients were grown without antibiotics as follows; L. lactis BU-260 in GM17 agar at 30°C and E. faecalis in BHI agar at 37°C. All strains were grown overnight. From the resulting culture, a 2% aliquot was used to inoculate fresh broth (without antibiotics) and incubated for 4 h to reach mid-exponential phase of growth. A cell suspension was recovered by centrifugation (7,426 × g for 10 min at 4°C), washed by resuspension and centrifugation in maximum recovery diluent (MRD) (CM733; Oxoid), and diluted in 3 ml MRD. Control cultures of donor and recipient bacteria were plated onto agar selective for donors, recipients, and transconjugants. Plates were incubated for 48 h at appropriate temperatures. All matings were repeated three times in duplicate.

Survival of LAB in rumen fluid. Rumen fluid was collected from two ruminally fistulated Frisian steers. One animal was fed a grass hay diet, and the other one was fed a wet silage diet; both diets were supplemented with vitamins and minerals. The steers were gradually adapted to the diets for 10 days prior to the collection of rumen fluid. Fluid was obtained by using a vacuum pump and was transported to the laboratory in a prewarmed (38°C) insulated thermos flask.

The rumen fluid was strained through two layers of muslin into sterile containers and maintained at 38°C until required. Half of the fluid obtained from each steer was centrifuged (7,426 × g for 10 min.) to reduce the number of resident bacteria. This resulted in four types of rumen fluid as follows: (i) raw rumen fluid from an animal on a diet of hay; (ii) centrifuged rumen fluid from an animal on the same diet of hay; (iii) raw rumen fluid from an animal on a wet silage diet; and (iv) centrifuged rumen fluid from the latter animal (wet silage diet).

LAB strains (1 × 10⁵ CFU ml⁻¹) were inoculated into each type of rumen fluid and maintained at 38°C over a 48-h period. After 48 h, the enumeration of LAB strains was completed by surface plating dilutions onto antibiotic-selective plates (antibiotic concentrations as per “Bacterial strains”; selective plates also contained cycloheximide (Sigma-Aldrich) to reduce yeast contamination. Rumen fluid with no LAB inoculants served as a control.

Mating in rumen model. Donor strains selected for inclusion in the rumen model were L. lactis SH4174, L. lactis 477, and S. thermophilus E2, and recipients chosen were L. lactis BU-260 and E. faecalis HJ2-2 (Table 1). A batch fermentation system was set up according to Tilley and Terry (33). Dried milled grass feed (0.5 g) was added to sterile 80- to 90-ml glass centrifuge tubes. A “synthetic saliva” buffer was prepared (33), and CO₂ gas was passed through the buffer while being heated to 38°C. Forty milliliters of this buffer solution was then added to each tube, followed by addition of 10 ml of rumen fluid. Tubes were flushed with CO₂ gas and sealed with a rubber cork fitted with a Bunsen gas release valve. Each tube was inoculated with 3 ml of donor culture and 3 ml of recipient culture prepared as described above (see “Inoculum preparation”). A control for each rumen fluid where 6 ml of sterile deionized water replaced the bacterial culture was carried out. All tubes were again flushed with CO₂ gas and incubated in a shaker table incubator (shaken incubation) or in a water bath (static incubation), set at 38°C.

At 0 (time model reached 38°C, 1 to 2 h approximately), 24, and 48 h after inoculation, samples (1 ml) were removed from each tube and dilutions spread plated onto selective agar for enumeration. All plates were incubated for 48 h. The experiment was repeated three times in duplicate.

Plant model mating. The donor strains used included L. lactis SH4174, L. lactis 477, and L. lactis 487, and the recipient strain used was L. lactis BU-260. The model (modified version of that of Molbø et al. [16]) consisted of 4 g of alfalfa sprouting seeds (Tamar Organics, The Organic Centre, Rossinver, Co. Leitrim, Ireland), which were aseptically weighed and placed in sterile plastic containers and covered. Seeds were inoculated with 3 ml of donor bacterial culture and 3 ml of recipient bacterial culture, as previously described above (see “Inoculum preparation”), and allowed to soak overnight at room temperature, at an average temperature of 22°C. A control was also set up whereby the seeds were soaked in 6 ml of sterile distilled water. The following day, the seeds were placed in

| TABLE 1. Bacterial strains used in this study |
|------------------------------------------------|
| Straina | Genotype(s) (location)¢ | Origin | Reference or source |
|------------------------------------------------|
| Lactococcus lactis SH4174b | 1erm(B) gene (pAM31 plasmid) | Food | 36 |
| Lactococcus lactis IB487b | 1tet(M) gene (transposon) | Dairy | IBB Poland (J. Bardowski, unpublished) |
| Lactococcus lactis IB487e | 1tet(M) gene (transposon) | Dairy | IBB Poland (J. Bardowski, unpublished) |
| Streptococcus thermophilus E2 5099b | 1erm(B) gene (plasmid) | Raw milk | 34 |
| Lactococcus lactis BU-260e | Strf Rif (chromosome, plasmid free) | Starter cultures | 19 |
| Enterococcus faecalis HJ2-2 | Fusf Rif (chromosome, plasmid free) | Clinical isolate | 12 |

¢ Sources: SH4174 and S0098, Istituto di Microbiologia, Università Cattolica, Piacenza, Italy; IB487 and IB487E, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland; LMG strains (BU-260 and HJ2-2), Laboratory of Microbiology and BCCM/LMG, Ghent University, Faculty of Sciences at Ghent University, Belgium.

a Donor strains.

b Recipient strains.

c Str, streptomycin; Rif, rifampin; Fus, fusidic acid.
stereile glass jars, capped with muslin, and placed at a 45° angle to allow for airflow and drainage. Seeds were watered twice daily with deionized water. On days 0, 1, 3, 6, and 9, after the seeds were placed in the sprouting jars, 10 sprouts or seeds were aseptically placed in stomacher bags containing 3 ml MRD and pulsed for 1 min, and dilutions were spread onto selective GM17 agar plates. For donor, recipient, and transconjugant enumeration, the same selective plates as outlined previously were used. All plates were incubated for 48 h. The experiment was repeated three times in duplicate.

**Plant mating controls.** A series of control experiments were set up in parallel with that of the plant model, designed to determine where transfer of antibiotic resistance takes place and if this transfer can occur independently of the seed system. To locate where the mating occurred, on the surface or within the germinating seed, the plant model experiment was set up as previously described, and after overnight incubation, seeds were either simply washed in MRD or pulsed in MRD in order to determine if mating can occur overnight regardless of seeds being present, the experiment was set up as described before but without seeds.

**Confirmation of transconjugants.** Presumptive transconjugants were cultured overnight in broth with antibiotic selection, then subsequently streaked onto agar plates containing antibiotics, and incubated at 30 or 37°C overnight. The stability of the antibiotic resistance marker was assessed by transferring colonies, after 40 h of growth in the absence of antibiotic pressure (first in broth [antibiotic free] overnight and subsequently on an agar plate [antibiotic free] overnight), onto selective antibiotic-containing agar plates. The stability test was repeated three times. The MIC of the transferred resistance gene was determined for both donor and transconjugant strains by applying an Etest strip (AB Biodisk, Solna, Sweden), according to the manufacturer’s instructions, to an inoculated LAB susceptibility test medium (35) agar plate. The MIC, defined by the intersection of the growth ellipse margin with the Etest strip, was read after overnight incubation.

**DNA extraction.** Genomic DNA was extracted from donors, recipients, and the presumptive transconjugants using a Qiagen DNeasy blood and tissue kit (catalog no. 69504; Qiagen Ltd., Crawley, West Sussex, United Kingdom), with modifications. Lysostaphin (0.5 mg ml⁻¹) (L7386; Sigma-Aldrich) and 200 U aprotinin were added to the enzymatic lysin buffer, and the incubation time was increased to 3 h. The concentration of DNA was determined by using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

**PCR detection of transferred genes.** PCR assays were performed to detect the corresponding resistance markers. The reaction mixture (total volume, 25 µl) consisted of 20 µmol of each primer, 1× Tag buffer (M0267S; New England Biolabs, Ipswich, MA), 0.2 mM deoxynucleoside triphosphate mix (U1511; Promega, Madison, WI), 0.5 U Taq DNA polymerase (M0267S; New England Biolabs, Inc.), and 100 ng purified DNA. Primers used for the detection of erm(B) were erm(B)-FW (5'-CAT TTA ACG ACA GAA CGG AC-3'), and erm(B)-RV (5'-GGA ACA TCT GTG GTA TGG CG-3') (8), and primers used for the detection of tet(M) were tet(M)-F (5'-GTG GAC AAA GGT ACA AGG AGA TAG-3') and tet(M)-R (5'-CGG TAA AGT TCG TCA CAT AC-3') (20). All PCR amplifications were performed in a Peltier thermal cycler (PTC-200; MJ Research, Inc., Watertown, MA) using the following cycling profile: an initial denaturation step at 94°C for 5 min; 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 10 min. PCR products (15 µl) were separated by electrophoresis on a 1% (wt/vol) agarose gel and visualized by ethidium bromide (10 mg ml⁻¹) staining.

**DNA fingerprinting by pulsed-field gel electrophoresis (PFGE).** (i) **Purification of genomic DNA.** A total of 10 ml of each cell culture was grown overnight to reach an optical density at 600 nm of 0.5 to 1.2, then centrifuged at 7,426 × g for 10 min to recover the cells, washed once in 10 ml 50 mM EDTA (pH 9.0) (Oxoid), and resuspended in the same solution. One hundred twenty-five micro-liters of the latter cell suspension was mixed with 750 µl of 1% (wt/vol) molten low-melting-point agarose prepared with 50 mM EDTA (pH 9.0). Inserts were prepared by dispensing 100 µl aliquots of the molten cell suspension into plug mold wells and allowing them to solidify at 4°C for 15 min. Cells were lysed by immersing the inserts in 3 ml 50 mM EDTA (pH 9.0) containing 2 mg ml⁻¹ lysozyme (Oxoid) and 0.05% (vol/vol) N-lauryl sarcosine (Oxoid) at 37°C, followed by overnight incubation at 50°C in 10% (vol/vol) sodium dodecyl sulfate (Oxoid), 10 mM Tris-HCl (pH 8.0) (Oxoid), and 50 mM EDTA (pH 9.0), containing 0.2% (wt/vol) proteinase K (Sigma-Aldrich). Inserts were subsequently washed five times by gentle shaking for 30 min at room temperature in 4 ml 50 mM EDTA (pH 9.0) and stored in this solution at 4°C. (ii) **Restriction enzyme treatment.** A total of 30 U Smal (New England Biolabs) restriction enzyme was added to 2-mm-long sections cut from agarose inserts and incubated at 25°C overnight. Digested genomic DNA in the agarose inserts was sealed into the wells of a 1.1% (wt/vol) PFGE (Bio-Rad, Richmond, CA) gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA). Bands were resolved by electrophoresis for 20 h at 14°C in a Chef-DR II PFGE unit with a model 1000 minchiller (Bio-Rad Laboratories, Hercules, CA). The gels were run at 6 V cm⁻¹ with an initial/final switch time of 3 s/80 s, respectively, and a linear ramping factor. A lambda ladder PFGE marker (N03408; New England Biolabs) was included as a size standard. Gels were stained in distilled water containing ethidium bromide (10 mg ml⁻¹) for 30 min, destained in 0.5× TBE, and photographed.

**Southern blotting and hybridization.** Genomic DNA was digested with the enzymes Smal and HindIII, and the fragments were separated on a 1% (wt/vol) agarose gel in Tris-acetate-EDTA buffer. The DNA fragments were transferred using upward capillary transfer to a Hybrid-N+ nylon membrane (RP203B; Amersham Life Sciences, Buckinghamshire, United Kingdom), and the membrane was hybridized with DNA probes constructed with erm(B) or tet(M), purified, and labeled PCR product, according to the manufacturer’s instructions (RP3000; GE Healthcare, Buckinghamshire, United Kingdom). The hybridizing fragments were visualized using the ECL detection system (GE Healthcare) and exposed to Hyperfilm ECL (RPZ2103K; GE Healthcare).

## RESULTS

**HGT rates observed using in vitro filter mating technique.** The filter mating technique was employed to assess the in vitro transfer frequencies of four LAB mating pairs. The average transfer frequencies obtained are shown in Table 2. High transfer frequencies were detected between all donor and recipient mating pairs. The highest transfer frequency was observed with *L. lactis* strain SH4174 (mating pair 1 [MP-1]), harboring pAMMj-1, followed by the *S. thermophilus* strain (MP-4), also harboring a plasmid, and then by the transposon containing *L. lactis* 487 (MP-3) and *L. lactis* 477 (MP-2).

**HGT rates observed using in vivo mating in rumeen model.** An initial study was carried out to examine the ability of LAB strains to survive in rumeen fluid over a 48-h period. Results obtained showed that all LAB donor and recipient strains survived in the rumeen fluid, regardless of the animal diet (hay/silage) or centrifugation of the fluid (data not shown).

Following the survival study, three LAB mating pairs were inoculated into the rumeen fluid model to examine their ability to transfer the genetic markers in this environment. Table 3 shows the mating conditions under which conjugal transfer occurred in the animal rumeen model, expressed as average transfer frequencies. Low, inconsistent transfer frequencies were observed, ranging from 1.0 × 10⁻⁹ (MP-4) to 8.0 × 10⁻⁶ (MP-2) transconjugants per recipient. The highest rate of transfer (at a frequency of 8.4 × 10⁻⁶ transconjugants/recipient) occurred with MP-2 using rumeen fluid from an animal fed a silage diet after 24 h of static incubation.

**HGT rates observed using in vivo mating in alfalfa plant model.** Three LAB mating pairs were inoculated into an alfalfa plant...
sprout model to assess transfer of the corresponding genetic resistance determinants in a model plant environment. The average transfer frequency rates obtained are shown in Table 4. High transfer frequencies were obtained between all three mating pairs in this model (transfer frequencies ranged from 2.7 × 10^{-4} to 3.4 × 10^{-1} transconjugants per recipient). Transfer frequency varied slightly over the 9-day testing period; MP-1 peaked at day 6, and MP-2 peaked at day 3, while the transfer frequency of MP-3 declined until day 3, after which it steadily increased to give the highest transfer frequency on day 9. The control experiment showed no transconjugants after overnight incubation without seeds present, indicating that seeds are involved in the mating occurrence (data not shown). No difference in transfer rates was detected when seeds were simply washed in MRD instead of pulsedified (data not shown), suggesting that transfer occurs mainly on the surface of the seed.

Confirmation of transconjugants. Colonies of presumptively erm(B) or tet(M) transconjugants were randomly picked from selective plates from independent mating experiments and tested in order to confirm that these isolates were true transconjugants and not reverted mutants. Transferred markers were shown to be stably maintained in all transconjugants in the absence of selective pressure. All donor/recipient control plates were negative, discounting the possibility of spontaneous mutation (the occurrence of a mutation within the genetic makeup of the strain, which would result in antibiotic resistance). Etest results for erythromycin and tetracycline showed that all the transconjugants displayed MICs of >256 µg ml^{-1} for the transferred marker, like those of the donor strains. The MICs for streptomycin were identical to those obtained by testing the parental streptomycin chromosomally resistant recipient strains (data not shown).

PCR analysis of the purified genomic DNA from the transconjugants gave positive signals for the presence of erm(B) or tet(M) genes (Fig. 1). The recipient strain was negative for these markers. PFGE DNA fingerprinting of transconjugant, donor, and recipient strains showed that the donor and recipient strains produced distinct SmaI PFGE patterns, while transconjugants displayed the DNA profiles of the corresponding recipient strains (Fig. 2).

Southern blotting analysis further confirmed the authenticity of the transconjugants by probe hybridization of the donors and transconjugants (data not shown).

**DISCUSSION**

Understanding the role of LAB in the dissemination of antimicrobial resistance determinants in the natural environment is important in terms of assessing their contribution to the spread of resistance through the food chain to the consumer. In this study, we reported on the ability of wild-type antibiotic

| TABLE 3. Conditions under which conjugal transfer occurred in the animal rumen model |
|---------------------------------|---------------------------------|-----------------|
| Mating pair (strains) | Mating conditions | Average transfer frequency |
|----------------|----------------|-----------------|
| MP-1 (L. lactis SH4174 + L. lactis BU-2-60) | Centrifuged, hay diet, static incubation, 0 h | 3.3 × 10^{-8} (0.000000057) |
| | Centrifuged, hay diet, static incubation, 48 h | 1.9 × 10^{-6} (0.000015) |
| | Centrifuged, silage diet, static incubation, 0 h | 1.9 × 10^{-8} (0.00000014) |
| MP-2 (L. lactis IBB477 + L. lactis BU-2-60) | Raw, silage diet, static incubation, 24 h | 8.0 × 10^{-6} (0.000005) |
| | Raw, silage diet, shaken incubation, 24 h | 5.0 × 10^{-8} (0.00000002) |
| | Centrifuged, hay diet, shaken incubation, 0 h | 4.6 × 10^{-8} (0.00000032) |
| MP-4 (S. thermophilus E2 + E. faecalis JH2-2) | Raw, hay diet, static incubation, 24 h | 4.0 × 10^{-9} (0.000000012) |
| | Raw, silage diet, static incubation, 24 h | 1.0 × 10^{-9} (0.0000000) |
| | Raw, silage diet, shaken incubation, 24 h | 3.6 × 10^{-8} (0.00000021) |
| | Centrifuged, hay diet, shaken incubation, 48 h | 3.6 × 10^{-8} (0.00000012) |
| | Centrifuged, silage diet, static incubation, 24 h | 1.3 × 10^{-8} (0.0000001) |
| | Centrifuged, silage diet, static incubation, 48 h | 3.0 × 10^{-8} (0.00000063) |
| | Centrifuged, silage diet, shaken incubation, 24 h | 1.5 × 10^{-8} (0.00000025) |
| | Centrifuged, silage diet, shaken incubation, 48 h | 3.2 × 10^{-8} (0.00000004) |

Transfer frequency was calculated as the number of transconjugants obtained per recipient strain. Values are the averages of three replications, with the standard deviations stated in parentheses.

| TABLE 4. Conjugal transfer on alfalfa plant model |
|----------------|----------------|-----------------|-----------------|-----------------|-----------------|
| Mating pair (strains) | Transfer frequency | Day 0 | Day 1 | Day 3 | Day 6 | Day 9 |
|----------------|----------------|-------------|-------------|-------------|-------------|-------------|
| MP-1 (L. lactis SH4174 + L. lactis BU-2-60) | 1.8 × 10^{-1} (0.12) | 1.1 × 10^{-1} (0.1) | 3.6 × 10^{-3} (0.42) | 3.9 × 10^{-3} (0.28) | 2.2 × 10^{-1} (0.19) |
| MP-2 (L. lactis IBB477 + L. lactis BU-2-60) | 1.2 × 10^{-3} (0.0014) | 2.8 × 10^{-3} (0.0038) | 6.6 × 10^{-3} (0.0066) | 2.8 × 10^{-3} (0.0028) | 8.5 × 10^{-4} (0.00085) |
| MP-3 (L. lactis IBB487 + L. lactis BU-2-60) | 1.6 × 10^{-3} (0.0012) | 1.4 × 10^{-3} (0.0016) | 4.7 × 10^{-4} (0.00055) | 1.6 × 10^{-3} (0.0023) | 3.5 × 10^{-3} (0.0046) |

Transfer frequency was calculated as the number of transconjugants obtained per recipient strain. Values are averages of three replications; standard deviations are stated in parentheses.
resistance determinants [erm(B) and tet(M)], present in LAB strains isolated from food sources, to be transferred to recipient strains. In vitro mating, using a traditional filter mating technique, showed that all four LAB mating pairs transferred their resistance determinants at high frequencies. Our study also explored the possibility of antibiotic resistance gene transfer occurring between LAB strains in modeled natural environments. By employing two in vivo models, an alfalfa sprout plant and an animal rumen model, we demonstrated the transfer of resistance determinants between all four LAB mating pairs in these models. Previously, in vivo transfer between LAB strains has only been shown in the gastrointestinal tracts of gnotobiotic rats (13) and mice (14, 17).

The in vitro transfer of pAMβ-1, in Lactococcus lactis SH4174, has been well documented and has already been shown to transfer via conjugation to different genera, including Bacillus, Clostridium, Staphylococcus, Enterococcus, Lactobacillus, and Lactococcus (2, 7, 10, 17, 27, 30, 31, 36). It was therefore chosen in this study as a model for conjugal transfer, and the transfer frequencies obtained were comparable to data obtained in previous studies. Previously, the in vitro transfer of a tet(M) resistance marker from two wild-type Lactococcus lactis strains, IBB477 and IBB487, to Lactococcus lactis BU-2-60 has been demonstrated by IBB Poland (J. Bardowski, personal communication and unpublished data). However, few studies examined the transfer of resistance genes from Streptococcus thermophilus. Wang et al. (37) reported that Streptococcus thermophilus was an important host of antibiotic resistance genes in cheese microbiota and transmitted its R-plasmid into streptococci using transformation methods.

Although an earlier study investigated the survival of LAB strains in rumen fluid (29) and another study investigated the possible probiotic effects of feeding ruminant animals silage and feeds inoculated with LAB strains (38), there have been no reports of transfer studies involving LAB strains carried out in the rumen fluid of animals. Indirect evidence showed that gene transfer between ruminal bacteria probably occurred, a process which appears to be continuous in this environment (18, 25). In our study, transfer of resistance genes from LAB strains in rumen fluid (29) and another study investigated the possible probiotic effects of feeding ruminant animals silage and feeds inoculated with LAB strains (38), there have been no reports of transfer studies involving LAB strains carried out in the rumen fluid of animals. Indirect evidence showed that gene transfer between ruminal bacteria probably occurred, a process which appears to be continuous in this environment (18, 25). 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Mizan et al. (15) reported that the cow’s rumen is a favorable environment for the genetic exchange of plasmids between *Escherichia coli* strains.

The highest transfer frequency obtained in our study occurred in the alfalfa sprout plant model (MP-1, 3.9 × 10⁻¹ transconjugants/recipient), indicating that this environment may be optimal for gene transfer. Each mating pair inoculated into the plant model transferred resistance at a higher frequency compared to the same matched pairs tested under in vitro conditions. It may be reasonable to suggest that the reason for the high numbers of transconjugants obtained in the plant model might include the fact that the surface of the seed or sprout may have acted to constrain the mobility of mating strains, maintaining them in intimate contact to promote conjugation. Mølbak et al. (16) also used alfalfa sprouts to examine plasmid transfer from *Pseudomonas putida* to indigenous plant bacteria and similarly reported high rates of transfer. In contrast to our findings, Mølbak et al. (16) did not observe transconjugants until day 6 and found that the transfer of plasmids took place preferentially in the root environment. We observed transconjugants from day 0, after an initial overnight soaking of the inoculum (containing donor and recipient strains) into the seeds. Perhaps the difference in the time of transfer initiation was due to the availability of recipients; for Mølbak et al. (16), indigenous recipient bacteria may not have been available for uptake of the plasmid into the seed, whereas in our study, both the donor and recipient were initially added to seeds in high concentrations, with transfer occurring on the seed surface. The work of Normander et al. (21) would concur with this observation, in that they observed transconjugant cells. Applied Environ. Microbiol. 57:2710–2713.

Although most food-associated LAB are generally regarded as safe to consume, the potential public health risk, arising from reports of the transfer of antibiotic resistance determinants from resistant LAB reservoir strains to commensal and pathogenic bacteria alike, needs to be investigated. Our results demonstrated that LAB strains containing antibiotic resistance-encoding genes can transfer these markers to other bacteria in modeled natural and laboratory-derived environments. These observations need to be carefully assessed, and the risks associated need to be considered, in light of the increasing concerns related to food as a potential reservoir for antibiotic resistance determinants.

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