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In vitro conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria

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Abstract

The ability of 14 *Lactobacillus* strains, isolated from fermented dry sausages, to transfer tetracycline resistance encoded by *tet*(M) through conjugation was examined using filter mating experiments. Seven out of 14 tetracycline-resistant *Lactobacillus* isolates were able to transfer in vitro this resistance to *Enterococcus faecalis* at frequencies ranging from 10^{-4} to 10^{-6} transconjugants per recipient. Two of these strains could also transfer their resistance to *Lactococcus lactis* subsp. *lactis*, whereas no conjugal transfer to a *Staphylococcus aureus* recipient was found. These data suggest that meat lactobacilli might be reservoir organisms for acquired resistance genes that can be spread to other lactic acid bacteria. In order to assess the risk of this potential hazard, the magnitude of transfer along the food chain merits further research.

Keywords: *Lactobacillus plantarum*; *Lactobacillus alimentarius*; *Lactobacillus sakei* subsp. *sakei*; Fermented dry sausage

1. Introduction

Lactobacilli are common in foods and are members of the resident microflora of the gastrointestinal tract of humans and animals. Because of their broad environmental distribution, these commensal bacteria may function as vectors for the dissemination of antibiotic resistance determinants via the food chain to the consumer [1]. In addition, this normal flora might be capable of supplying antibiotic resistance genes to food-borne or enteric pathogens [2]. Although plasmids are very common in lactobacilli [3] and plasmid-located antibiotic resistance determinants have been reported in lactobacilli [4–10], literature on the conjugal transfer of native *Lactobacillus* plasmids is limited. In this context, the conjugal transfer of plasmid-encoded lactose metabolism from *Lactobacillus casei* [11], and of bacteriocin production and resistance from *Lactobacillus acidophilus* [12] has been reported before.

In a previous study, we found that strains of several *Lactobacillus* species isolated from fermented dry sausages harboured the tetracycline resistance gene *tet*(M) [13]. In most cases, this gene was located on plasmids of different sizes (from 7 to more than 30 kb) and displayed very high sequence similarities with *tet*(M) genes previously reported in the pathogenic species *Neisseria meningitidis* and *Staphylococcus aureus*. As a follow-up to the latter paper, the current study was set out to investigate the potential of these tetracycline-resistant (Tc^r) *Lactobacillus* isolates to transfer the *tet*(M) gene to other Gram-positive bacteria, including *Enterococcus faecalis*, *Lactococcus lactis* subsp. *lactis* and *Staphylococcus aureus*.

2. Materials and methods

2.1. Bacterial strains

The strains used in this study are listed in Table 1. The Tc^r lactobacilli, used as donor strains for mating experiments, were isolated from fermented dry sausage end products as described previously [13], and grown on MRS (0882210, Becton Dickinson, Franklin Lakes, MD, USA) at 30°C. The following recipient strains were used: (i) *E. faecalis* JH2-2 [14] was grown in brain heart infusion

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medium (Becton Dickinson) at 37°C; (ii) the lactose-negative *L. lactis* subsp. *lactis* Bu2-60 [15] was grown in M17 broth medium (CM0817, Oxoid, Basingstoke, UK) in which lactose was replaced by glucose (GM17) at 30°C; and (iii) *S. aureus* 80CR5 [16] was grown in nutrient broth (CM0001, Oxoid) at 37°C. All strains were stored in a bead storage system (Microbank system, Pro-LAB Diagnostics, Wirral, UK) at −80°C.

2.2. Mating procedure

Transferability of tetracycline resistance was examined by filter mating. For this purpose, donor and recipient strains were grown in non-selective broth medium to the mid-exponential phase of growth (approx. 4 h). The donor culture (1 ml) was added to the recipient culture (1 ml) and the mixture was filtrated through a sterile mixed cellulose esters filter (0.45 μm) (MF-Millipore membrane filter, HAWP 02500, Millipore, Bedford, MA, USA) using Swinnex® filter holders (SX00 025 00, Millipore). After donor and recipient cells were filtrated, sterilised peptone physiological saline solution (PPS) (8.5 g l^{-1} NaCl and 1 g l^{-1} neutralised bacteriological peptone [LP0034, Oxoid]) was passed through the filter to trap the cells more tightly into the membrane, according to Sasaki and co-workers [17]. The filters were incubated overnight on non-selective agar medium according to the optimal growth conditions of the recipient strain (see Section 2.1). The bacteria were washed from the filters with 2 ml PPS. Dilutions of the mating mixtures were spread onto agar plates containing 10 μg ml^{-1} tetracycline (Sigma, Bornem, Belgium) and 50 μg ml^{-1} rifampicin (Sigma) (double selective medium) and incubated for 24–48 h. Control cultures of donor and recipient strains were also individually plated on the double selective agar plates.

2.3. Antibiotic susceptibility testing and MIC determination

Possible transconjugants were screened for their antibiotic resistance pattern, using a modified version of the Kirby–Bauer disc diffusion method [18], in which Mueller–Hinton medium was replaced by MRS agar. The minimum inhibitory concentration (MIC) of tetracycline was determined by applying an Etest® strip (AB Biodisk, Solna, Sweden) on an inoculated MRS plate according to the manufacturer's instructions. The Etest was read following 16–18 h incubation at 30°C.

2.4. DNA preparation and manipulations

Total genomic DNA of each isolate was extracted and purified as described previously [19]. Isolation of plasmid DNA was based on the alkaline lysis method of Anderson and McKay [20]. Agarose gel electrophoresis and Southern blotting were carried out following standard procedures [21]. Labelling of DNA probes with horseradish peroxidase using the ECL Direct Nucleic Acid Labelling kit (RPN3000, Amersham Biosciences, Uppsala, Sweden) was performed according to the manufacturer's instructions.

2.5. Typing of transconjugants

The fingerprints of transconjugants, obtained by high-resolution $(GTG)_5$ polymerase chain reaction (PCR) fingerprinting [19], were compared to the fingerprints of recipient strains for confirmation purposes.

2.6. PCR detection of tet(M) and erm(B) genes

PCR assays were performed as described previously [13]. In brief, each PCR reaction (total volume, 50 μl) contained 20 pmol of each primer, 1×PCR buffer (Applied Biosystems, Warrington, UK), each of four dNTPs at a concentration of 200 μM, and 1 U of AmpliTaq DNA polymerase (N808-0160, Applied Biosystems). Primers used for detection of *tet*(M) were DI (5′-GAYACNCCNGGNCAYRTNGAYTT-3′) and tetM-R (5′-CACCGAGCAGGGATTTCTCCAC-3′), and for detection of *erm*(B) the primers were ermB-FW (5′-CATTTAACGACGAAACTGGC-3′) and ermB-RV (5′-GGAACATCTGTGGTATGGCG-3′). A 50-ng portion of purified total DNA was used as a template. All PCR amplifications were performed in a GeneAmp 9600 PCR system (Perkin-Elmer) using the following temperature programme: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°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 visualised by ethidium bromide staining.

3. Results

A total of 14 Tc^r *Lactobacillus* isolates (Table 1) all containing a *tet*(M) gene were used to test their ability to transfer tetracycline resistance to *E. faecalis* JH2-2, *L. lactis* subsp. *lactis* Bu2-60 and *S. aureus* 80CR5 by conjugation. Several attempts to obtain transconjugants by filter mating using a 0.2-μm pore size filter were ineffective (data not shown), whereas the use of a 0.45-μm membrane with a sponge-like structure was more successful. Matings with *E. faecalis* JH2-2 as a recipient strain were successful for seven out of 14 donor strains, including four *Lb. plantarum* strains (DG 013, DG 507, DG 515 and DG 522), two *Lactobacillus alimentarius* strains (DG 498 and DG 500), and one *Lactobacillus sakei* subsp. *sakei* strain (DG 493) at frequencies ranging between 10^{-4} and 10^{-6} transconjugants per recipient. Highest transfer frequencies were found when cells were grown until the mid-exponential phase (4–6 h) in comparison to overnight cultures. Two out of 14 Tc^r *Lactobacillus* isolates (DG 493 and DG 515) could also transfer tetracycline resistance to

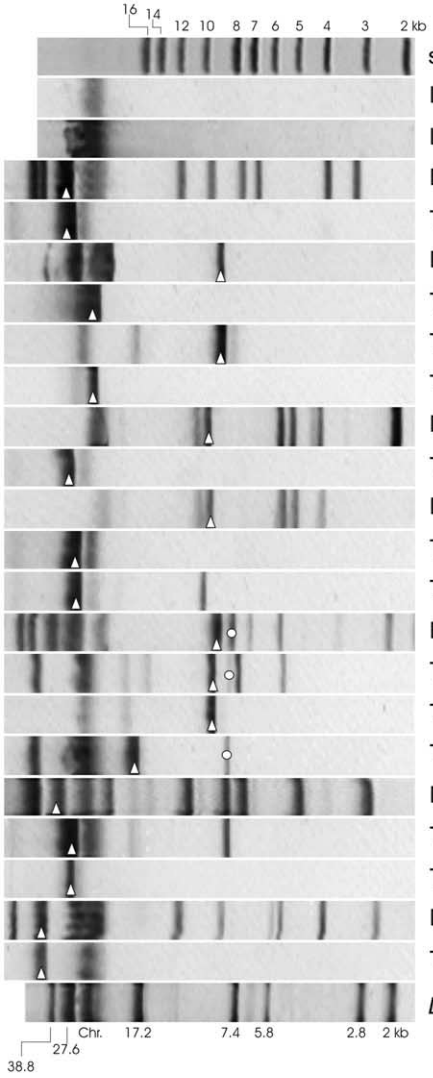
Table 1
Bacterial strains used in this study

Strain	Relevant properties	Remarks	References
Lactobacillus plantarum			
DG 013 (LMG 21677), DG 509 (LMG 21685), DG 512 (R-12511), DG 515 (LMG 21686), DG 522 (LMG 21687)	Plasmid-located *tet*(M) gene		
DG 507 (LMG 21684)	*tet*(M) and *erm*(B) genes on two different plasmids		
Lactobacillus curvatus			
DG 142 (LMG 21679), DG 484 (LMG 21681), DG 524 (LMG 21688)	Chromosomally located *tet*(M) gene	Donor strains, Source: fermented dry sausages	[13]
Lactobacillus alimentarius			
DG 498 (R-12497), DG 500 (LMG 21683)	Plasmid-located *tet*(M) gene		
Lactobacillus sakei subsp. *sakei*			
DG 493 (LMG 21682)	Plasmid-located *tet*(M) gene		
Lactobacillus sakei subsp. *carnosus*			
DG 048 (LMG 21678), DG 165 (LMG 21680)	Plasmid-located *tet*(M) gene		
Enterococcus faecalis			
JH2-2 (LMG 19456)	Fus^r, Rif^r, plasmid-free	Recipient strain	[14]
Lactococcus lactis subsp. *lactis*			
Bu2-60 (LMG 19460)	Str^r, Rif^r, plasmid-free	Recipient strain	[15]
Staphylococcus aureus			
80CR5 (LMG 21674)	Fus^r, Rif^r, Pen^r	Recipient strain	[16]
Lactococcus lactis subsp. *cremoris*			
AC1	Used as plasmid size marker		[15]

Fus: fusidic acid; Rif: rifampicin; Str: streptomycin; Pen: penicillin.
Strains with LMG number were deposited in the BCCM®/LMG Bacteria Collection (http://www.belspo.be/bccm/lmg.htm).

L. lactis subsp. *lactis* Bu2-60 at frequencies ranging between 10^{-5} and 10^{-7} transconjugants per recipient. No transconjugants could be obtained after matings of the 14 Tc^r *Lactobacillus* isolates with *S. aureus* 80CR5 as recipient strain. Potential transconjugant colonies (approximately five per mating experiment) were isolated from the double selective medium (containing both 10 μg ml^{-1} tetracycline and 50 μg ml^{-1} rifampicin) at the end of the filter mating experiment, purified on non-selective medium and checked for coccoid cell morphology using standard phase-contrast microscopy. Using disc diffusion testing, susceptibility to tetracycline and rifampicin was compared between donor (Tc^r/Rif^s phenotype), recipient (Tc^s/Rif^r phenotype) and transconjugants (Tc^r/Rif^r phenotype). All selected Tc^r cocci displayed the Tc^r/Rif^r phenotype. Further confirmation of the transconjugant's identity was obtained by comparing the $(GTG)_5$ PCR fingerprints of donor, recipient and corresponding transconjugants, and by checking the presence of the *tet*(M) gene by PCR. On the basis of these criteria, all Tc^r cocci that were isolated from the double selective medium were confirmed as true transconjugants.

Genotypic characterisation of the transferred plasmids was obtained by plasmid profiling in combination with Southern blotting and hybridisation. In most cases, all transconjugants resulting from a particular donor/recipient combination exhibited the same plasmid profile, and from those, only one transconjugant was selected for blotting and hybridisation experiments. Among transconjugants obtained from mating of donor strains DG 493, DG 500 and DG 507 with the *E. faecalis* JH2-2 recipient strain, however, more than one different plasmid profile per combination was found. In these cases, one strain for each different plasmid profile was selected. A total of 13 transconjugants from nine different donor/recipient combinations were selected for blotting and hybridisation experiments, thereby selecting towards a representation of the maximum in plasmid profile diversity (Fig. 1). In six transconjugants, the plasmid band that hybridised with the *tet*(M) probe was different in size compared to the original R-plasmid of the donor strain. Next to the plasmid of approx. 10 kb encoding tetracycline resistance, two out of three transconjugants from the matings with DG 507 as donor strain also received a second plasmid (approx. 8.5 kb) containing an *erm*(B) gene, as confirmed by PCR. This was also reflected in the MICs for erythromycin, which increased from 1 μg ml^{-1} for the erythromycin-susceptible transconjugants to >256 μg ml^{-1} for those that received the plasmid containing the *erm*(B) gene (Fig. 1). The MICs for tetracycline of the *E. faecalis* JH2-2 transconjugants were at least three times lower than the MIC of the corresponding donor strain, whereas the MICs of the



Strain No.	Transfer frequency	Taxon	MIC of tetracycline	MIC of erythromycin
supercoiled DNA ladder, Gibco BRL (plasmid size marker)				
LMG 19456		*E. faecalis* JH2-2 (recipient)	0.5	1
LMG 19460		*Lc. lactis* Bu2-60 (recipient)	0.19	
DG 013		*Lb. plantarum*	>256	
TC 013 - 1	$6.5\ 10^{-5}$	*E. faecalis* JH2-2	96	
DG 493		*Lb. sakei* subsp. *sakei*	192	
TC 493 - 1	$1.0\ 10^{-4}$	*E. faecalis* JH2-2	64	
TC 493 - 4		*E. faecalis* JH2-2	64	
TC 493 - 21	$1.3\ 10^{-5}$	*Lc. lactis* Bu2-60	192	
DG 498		*Lb. alimentarius*	>256	
TC 498 - 1	$2.9\ 10^{-4}$	*E. faecalis* JH2-2	96	
DG 500		*Lb. alimentarius*	>256	
TC 500 - 1	$9.0\ 10^{-5}$	*E. faecalis* JH2-2	64	
TC 500 - 3		*E. faecalis* JH2-2	64	
DG 507		*Lb. plantarum*	>256	>256
TC 507 - 1		*E. faecalis* JH2-2	64	>256
TC 507 - 2	$3.3\ 10^{-5}$	*E. faecalis* JH2-2	64	1
TC 507 - 4		*E. faecalis* JH2-2	64	>256
DG 515		*Lb. plantarum*	>256	
TC 515 - 1	$1.0\ 10^{-6}$	*E. faecalis* JH2-2	64	
TC 515 - 21	$6.4\ 10^{-7}$	*Lc. lactis* Bu2-60	>256	
DG 522		*Lb. plantarum*	>256	
TC 522 - 1	$1.0\ 10^{-4}$	*E. faecalis* JH2-2	64	
Lc. lactis subsp. *cremoris* AC1 (plasmid size reference)				

Fig. 1. Plasmid profiles of the donor (DG), recipient (LMG) and transconjugants (TC) and plasmid size reference. Results of Southern hybridisation analysis with the *tet*(M)/*erm*(B) probes are indicated with small triangles/circles respectively. Colours of gel were digitally inverted for improved band recognition. *L. lactis* subsp. *cremoris* strain AC1 was used as a plasmid size marker [15]. MIC was determined by Etest; Chr.: chromosomal band; transfer frequency: number of transconjugants/number of recipient cells as determined by plate counts.

L. lactis Bu2-60 transconjugants were comparable to those of the corresponding donor strain.

4. Discussion

The filter mating experiments described in this study demonstrate that intergeneric transfer of R-plasmids from meat *Lactobacillus* spp. to other lactic acid bacteria, including *Enterococcus* and *Lactococcus*, can occur at high frequencies under laboratory conditions of intimate cell-to-cell contact. From the methodological point of view, two factors seemed to significantly affect the transfer frequency, namely the type of membrane filter (type, pore size and side of membrane) and the age of donor and recipient cultures. Similar filter-dependent transfer frequencies have been reported before by Sasaki and co-workers [17], who indicated that the use of a sponge-like membrane with a pore size of 0.45 μm and front side up resulted in the highest transfer frequencies. Moreover, they indicated that these frequencies could be increased when cells were trapped more tightly in the spongy structure of the membrane by passing sterile water or buffer through the filter. Langella and co-workers observed that conjugal transfer of pAMβ1 between *Lb. sakei* strains is also possible on solid surface agar, although at lower frequencies [22].

The host range of the *tet*(M)-containing R-plasmids was clearly variable and appeared to be limited to members of the lactic acid bacteria (*Enterococcus* and *Lactococcus*). Recently, the complete sequence of a *tet*(M)-containing plasmid from a *Lb. plantarum* strain was determined,

which showed a high sequence homology of its replicon with that of a *Tetragenococcus halophilus* plasmid [10]. The host range of the latter plasmid was previously shown to include the genera *Pediococcus*, *Enterococcus*, *Lactobacillus* and *Leuconostoc* but not the genus *Lactococcus* [23]. Our findings indicate that the R-plasmids of the investigated Tc^r *Lactobacillus* strains have different conjugation abilities: some plasmids were transferable to the genera *Enterococcus* and *Lactococcus* (DG 493, 515), some to *Enterococcus* (DG 013, 498, 500, 507, 522), and others to none of the three recipient strains (DG 048, 165, 509, 512). Based on our conjugation experiments with only one *S. aureus* strain, we cannot exclude this species from the host range.

In a few transconjugants (TC 500-3, TC 507-1, TC 507-4 and TC 515-1), additional plasmids, other than the plasmid encoding the tetracycline resistance, seemed to have co-transferred spontaneously. This resulted for example in co-transfer of the erythromycin resistance determinant *erm*(B) from strain DG 507 into *E. faecalis*. The widespread distribution of tetracycline resistance, and the *tet*(M) gene in particular, which resulted in a reduced effectiveness and consequently in a reduced usefulness of tetracyclines, limits the significance of our findings regarding human safety. Erythromycin resistance, on the other hand, is much more rare, making the spontaneous co-transfer hugely significant regarding the effectiveness of macrolides, nowadays still important antibiotics in fighting human infections. Therefore, extensions to our choice for tetracycline resistance as a model system towards other resistances can be suggested as a highly relevant topic for further research.

Remarkably, in six of the investigated transconjugants the band that hybridised with the *tet*(M) probe displayed a different size than the R-plasmid of the donor strain. These bands were two (TC 507-4) to three (TC 493-1, TC 493-21, TC 498-1, TC 500-1 and TC 500-3) times the size of the R-plasmid of the donor strain. So far, no further research has been undertaken to elucidate this finding. In the transconjugants TC 493-1 and TC 493-21, the band that hybridises with the *tet*(M) probe coincides with the chromosomal band, which might suggest a chromosomal integration of the resistance determinant. However, location of the *tet*(M) gene on a plasmid that migrates at the same height as the chromosomal band cannot be excluded.

To our knowledge, this is the first report demonstrating the in vitro conjugal transfer of native *Lactobacillus* plasmids encoding an antibiotic resistance determinant to other lactic acid bacteria. A few studies have shown the transfer of an introduced plasmid, such as pAMβ1 (encoding erythromycin resistance) from *Lb. reuteri* and *Lb. plantarum* to other Gram-positive bacteria in vitro [24,25] and in vivo [26]. The in vivo transfer rate of pAMβ1 increased from 10^{-7} to 10^{-4} when erythromycin selective pressure was applied [26]. The mobilisation of a non-conjugative, native plasmid encoding chloramphenicol resistance from *Lb. plantarum* to *Carnobacterium piscicola* was achieved by co-mobilisation with the conjugative plasmid pAMβ1 [6]. Likewise, lactobacilli have also been extensively studied as plasmid recipients, such as for the broad-host-range plasmid pAMβ1 in the framework of optimising recombinant DNA technologies to improve strain properties as reviewed by Wang and Lee [3]. In this context, Reniero and co-workers reported that the production of an aggregation-promoting protein stimulated the uptake of pAMβ1 in *Lb. plantarum* strain with transfer frequencies as high as 10^{-2} using solid matings [28].

When considering all current evidence on their donor and recipient potential, it can be suggested that food *Lactobacillus* spp. can act as reservoir organisms of acquired antibiotic resistance genes that can be disseminated to other bacteria, a possibility that so far has only been poorly addressed. Vogel and co-workers [27] demonstrated that conjugal transfer of plasmid DNA (e.g. pAMβ1) can occur among starter lactobacilli during in situ sausage fermentation at frequencies in the same order of magnitude as in vitro, i.e. 10^{-6} transconjugants per recipient. Although the findings of this study indicate a potential food safety hazard, the magnitude of the risk is yet to be established in a risk assessment. Such an analysis will require more in-depth research on the prevalence and molecular basis of acquired antibiotic resistance in non-pathogenic bacteria, characterisation of the R-plasmids and in vitro and in vivo transferability of these resistances to pathogenic bacteria.

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