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ORIGINAL ARTICLE

Identification and characterization of antibiotic resistance genes in *Lactobacillus reuteri* and *Lactobacillus plantarum*

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Keywords
antibiotic resistance, *erm* genes, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *tet* genes.

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Abstract

Aims: The study aimed to identify the resistance genes mediating atypical minimum inhibitory concentrations (MICs) for tetracycline, erythromycin, clindamycin and chloramphenicol within two sets of representative strains of the species *Lactobacillus reuteri* and *Lactobacillus plantarum* and to characterize identified genes by means of gene location and sequencing of flanking regions.
Methods and Results: A *tet*(W) gene was found in 24 of the 28 *Lact. reuteri* strains with atypical MIC for tetracycline, whereas four of the six strains with atypical MIC for erythromycin were positive for *erm*(B) and one strain each was positive for *erm*(C) and *erm*(T). The two *Lact. plantarum* strains with atypical MIC for tetracycline harboured a plasmid-encoded *tet*(M) gene. The majority of the *tet*(W)-positive *Lact. reuteri* strains and all *erm*-positive *Lact. reuteri* strains carried the genes on plasmids, as determined by Southern blot and a real-time PCR method developed in this study.
Conclusions: Most of the antibiotic-resistant strains of *Lact. reuteri* and *Lact. plantarum* harboured known plasmid-encoded resistance genes. Examples of putative transfer machineries adjacent to both plasmid- and chromosome-located resistance genes were also demonstrated.
Significance and Impact of the Study: These data provide some of the knowledge required for assessing the possible risk of using *Lact. reuteri* and *Lact. plantarum* strains carrying antibiotic resistance genes as starter cultures and probiotics.

Introduction

The emergence of antibiotic-resistant bacteria is a well-known problem primarily caused by the excessive and inappropriate use of antibiotics in human and veterinary medicine, animal husbandry, agriculture and aquaculture (Tenover and Hughes 1996). Lactic acid bacteria (LAB) have a long history of safe use as food-processing aids, and as probiotics, they are associated with health benefits (Pham *et al.* 2008). However, when present in the food chain and in the intestinal tract of animals and humans, these bacteria may function as reservoirs of mobile antibiotic resistance genes that can be transferred to pathogenic bacteria (Teuber *et al.* 1999; Salyers *et al.* 2004).

Lactobacillus reuteri and *Lactobacillus plantarum* are LAB occurring naturally in the gastrointestinal, vaginal and oral tract of humans and warm-blooded animals. *Lactobacillus plantarum* is commonly used as a starter inoculant for the fermentation of food and feed of plant origin, including silage (Hammes and Hertel 2006). Both species are associated with lactic acid fermentation of sourdough (Vogel *et al.* 1994), and they are also used as probiotics (Casas and Dobrogosz 2000; Hammes and Hertel 2006). Information on the genetic mechanisms generating reduced antibiotic susceptibility in these species is limited, although plasmid-encoded antibiotic resistance genes have been reported in a few strains of both *Lact. reuteri* (Vescovo *et al.* 1982; Axelsson *et al.* 1988; Tannock *et al.* 1994; Lin *et al.* 1996) and *Lact. plantarum* (Danielsen 2002; Gevers *et al.* 2003a).

In previous studies, we determined the normal distribution of antibiotic minimum inhibitory concentrations

(MICs) in *Lact. reuteri* and *Lact. plantarum* by for each species testing a representative set of strains. A bimodal distribution of MICs was obtained for tetracycline, erythromycin, clindamycin, ampicillin and chloramphenicol in *Lact. reuteri* strains from various human and animal habitats (Egervärn *et al.* 2007a), and for tetracycline in *Lact. plantarum* strains isolated from fermented plant products (Flórez *et al.* 2006). Comparison of MICs and repetitive DNA element PCR (rep-PCR) genomic fingerprinting data revealed genetic relatedness among a group of *Lact. reuteri* strains with high MIC for tetracycline and among strains with high MICs for both erythromycin and clindamycin. Our study aimed to identify the resistance genes in *Lact. reuteri* and *Lact. plantarum* mediating atypical MICs for tetracycline, erythromycin, clindamycin and chloramphenicol. In addition, location (plasmid or chromosome) and DNA sequence of flanking regions of identified resistance genes were determined.

Materials and methods

Bacterial strains

All 36 *Lact. reuteri* strains were selected based on MIC data previously reported by Egervärn *et al.* (2007a). This included the 32 *Lact. reuteri* strains with MICs above 64 μg ml^{-1} for tetracycline and/or above 256 μg ml^{-1} for erythromycin, one strain (5010) with MIC of 128 μg ml^{-1} for chloramphenicol and three strains (BR11, ATCC PTA5289 and ATCC PTA6127) with MICs in the lower test range, but isolated from the same host and/or displaying rep-PCR fingerprints similar to strains with atypical tetracycline or chloramphenicol MICs (Fig. 1). The strains were obtained from BioGaia AB (Stockholm, Sweden; $n = 25$), the Department of Microbiology, Swedish University of Agricultural Sciences (Uppsala, Sweden; $n = 7$), the Chr. Hansen Culture Collection (Hørsholm, Denmark; $n = 3$) and the BCCM/LMG Bacteria Collection, Ghent University (Ghent, Belgium; $n = 1$). The strains were originally isolated from humans ($n = 15$), pigs ($n = 6$), rodents ($n = 4$), birds ($n = 4$), cows ($n = 4$), dogs ($n = 2$) and monkeys ($n = 1$). The human strains were isolated from intestine or faeces ($n = 5$), breast milk ($n = 5$), vagina ($n = 4$) and saliva ($n = 1$). Species confirmation was conducted by 16S rDNA sequence analysis and subtyping by rep-PCR genomic fingerprinting using the primer $(GTG)_5$, as previously reported (Egervärn *et al.* 2007a).

The two *Lact. plantarum* strains, included VTT E-042708 and VTT E-042709, displayed MIC above 256 μg ml^{-1} for tetracycline (Flórez *et al.* 2006). The strains were obtained from VTT Biotechnology and Food Research (Espoo, Finland) and were originally isolated from silage in Italy. Species confirmation was performed by a species-specific PCR and subtyping by rep-PCR genomic fingerprinting using the primer $(GTG)_5$, as previously reported (Flórez *et al.* 2006).

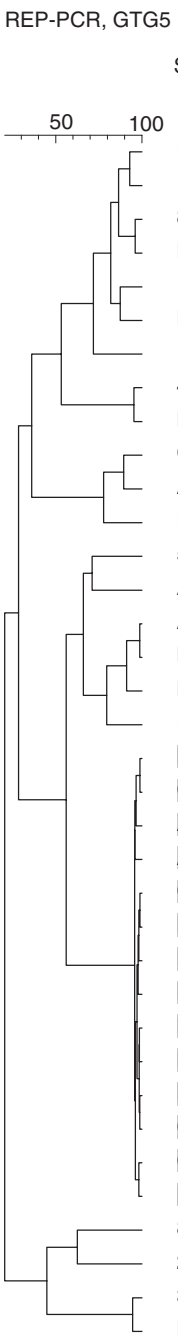
Detection of resistance genes

Bacteria were grown overnight on MRS agar (Oxoid, Basingstoke, UK) at 37°C in an anaerobic atmosphere (AneroGen; Oxoid). Total DNA was extracted with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR with specific primer pairs (Table 1) was used to detect the tetracycline resistance genes *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S) and *tet*(W), the erythromycin resistance genes *erm*(A), *erm*(B), *erm*(C), *erm*(T) and *mef*(A), and a chloramphenicol resistance gene *cat*-TC. The real-time PCR mixture was prepared in a 25-μl volume containing 1× SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA), 0·1 μmol l^{-1} of each primer and approx. 10 ng template DNA. DNA amplification was performed at the following conditions: 10 min at 95°C, 40 cycles with 15 s at 95°C, 30 s at 55°C and 45 s at 72°C. After amplification, the products were analysed by melting curve analysis to check amplification specificity. All real-time PCR reactions were performed using an ABI 7500 instrument (Applied Biosystems), and the data obtained were analysed with the 7500 System Sequence Detection ver. 1.3.1 software (Applied Biosystems). A 10-fold dilution series (10–0·001 ng) of a positive control for the *tet* and *erm* genes was included in each assay (Table 1). The resulting PCR product was in some cases purified with the QIAquick PCR Purification kit (Qiagen) and DNA sequenced at the Uppsala Genome Center (Uppsala, Sweden), using the forward primer. The sequences obtained were subjected to a Blast search in the GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA).

The DNA microarray assay was performed at RIKILT, Institute of Food Safety (Wageningen, the Netherlands), as described by van Hoek and Aarts (2008). The microarray contained various oligonucleotides specific for 33 oxy/tetracycline resistance genes and 25 chloramphenicol resistance genes.

Plasmid isolation

Selected bacterial strains were grown overnight in MRS broth at 37°C, then re-inoculated in 5 ml of the same medium and collected by centrifugation while exponentially growing (OD_{600} of 0·6–0·8). Plasmid DNA was extracted using QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions, with the



Strain	Host	MICs TET	MICs ERM	Resistance genes	Gene location	$\Delta\Delta C_t$
PA-16	pig	>256	>256	*tet*(W); *erm*(C)	chr*; plasmid	0·5; 3·8
100-67	chicken, intestine	4	>256	neg. ; *erm*(T)	nt ; plasmid	nt ; 6·1
8557:1	human, intestine	32	>256	neg. ; *erm*(B)	nt ; plasmid	nt ; 5·8
LpA1	pig	>256	1	*tet*(W); neg.	chr ; nt	0·6; nt
1068	pig, intestine	16	>256	neg. ; *erm*(B)	nt ; plasmid	nt ; 4·4
LMG 18391	pig, intestine	>256	>256	*tet*(W); *erm*(B)	plasmid*; plasmid	6·0; 5·0
1048	pig, intestine	16	>256	*erm*(B)	plasmid	6·1
4000	mouse	128	0·5	*tet*(W)	plasmid	7.8
BR11	guinea pig	32	1	neg.	nt	nt
Cow 10	cow, milk	>256	1	ND	nt	nt
ATCC PTA5289	human, saliva	16	2	neg.	nt	nt
DSM 20015	cow, manure	128	0·25	*tet*(W)	chr*	0·9
5010	dog, faeces	16	2	*tet*(W) ; cat-TC	chr ; plasmid	0 ; 4·5
ATCC PTA6127	dog, faeces	16†	0·5	neg.	nt	nt
ATCC 55148	chicken	256	1	ND	nt	nt
MF2-3	human, faeces	256	1	ND	nt	nt
MF14-C	human, faeces	256	0·5	ND	nt	nt
F14	pig	>256	1	*tet*(W)	plasmid	4·2
M45R2	human, breast milk	>256	1	*tet*(W)	plasmid	5·1
Cow 14	cow, milk	>256	1	*tet*(W)	nt	nt
ATCC 55730	human, breast milk	>256	1	*tet*(W)	plasmid*	7·9
ATCC 55149	turkey	>256	0·5	*tet*(W)	nt	nt
CF8-0A	human child, faeces	>256	2	*tet*(W)	nt	nt
MV6-2A	human, vagina	>256	2	*tet*(W)	plasmid*	8·1
MV14-1A	human, vagina	>256	1	*tet*(W)	nt	nt
MV29-2A	human, vagina	>256	1	*tet*(W)	nt	nt
M27U15	human, breast milk	>256	1	*tet*(W)	nt	nt
M81R43	human, breast milk	>256	0·5	*tet*(W)	nt	nt
MV4-1A	human, vagina	>256	2	*tet*(W)	nt	nt
Cow 18	cow, milk	>256	1	*tet*(W)	nt	nt
CF48-3A1	human child, faeces	>256	1	*tet*(W)	plasmid*	7·9
MM36-1A	human, breast milk	>256	1	*tet*(W)	plasmid	5·8
82	monkey	>256	2	*tet*(W)	plasmid	4·2
2010	rat	128	1	*tet*(W)	chr	–0·5
3040	hamster	>256	0·5	*tet*(W)	chr*	0·1
KS12	chicken	>256	1	*tet*(W)	chr	0·5

Figure 1 Dendrogram based on the $(GTG)_5$-PCR-generated genomic fingerprints of 36 selected *Lactobacillus reuteri* strains from different origins based on a previous study of antibiotic susceptibility in heterofermentative lactobacilli (Egervärn *et al.* 2007a). Minimum inhibitory concentrations (MICs) (μg ml^{-1}) of tetracycline (TET) and erythromycin (ERM) were determined by Etest. Gene location was determined by Southern hybridization (*) and a real-time PCR method in terms of $\Delta\Delta C_t$ values. A $\Delta\Delta C_t$ value of above 3·7 implied that the resistance gene was plasmid encoded. The shaded fields indicate a genotypic group.†The previously reported MIC = 128 μg ml^{-1} (Egervärn *et al.* 2007a) was incorrect and has been changed. chr, chromosome; ND, not detected; NT, not tested.

following exception. In the first step, 20 mg ml^{-1} lysozyme (Sigma-Aldrich, St Louis, MO, USA) and 100 U ml^{-1} mutanolysin (Sigma-Aldrich) were added to buffer P1, and the suspensions were incubated at 37°C for 2 h. Extracted plasmids were separated by electrophoresis in 0·8% agarose gel in 1× Tris–Phosphate–EDTA buffer. Gels were stained with ethidium bromide, and digitalized images were captured under UV light transillumination.

Table 1 Primers for PCR detection of selected antibiotic resistance genes

Genes	Forward primer	Reverse primer	Amplicon length (bp)	Positive control*	Reference
tet(K)	5′-GTAGCGACAATAGGTAATAG	5′-GCAACTTCTTCTTCAGAAAG	278	*Staphylococcus aureus* 1880/04	A. van Hoek, personal communication
tet(L)	5′-GTTTCGGGTCGGTAATTGGG	5′-GCTATCATTCCACCAATCGC	220	*Enterococcus faecium* 5878	A. van Hoek, personal communication
tet(M)	5′-ACACGCCAGGACATATGGAT	5′-CTTTTCCGCAAAGTTCAGAC	537	*Lactobacillus plantarum* 5057	A. van Hoek, personal communication
tet(O)	5′-AACTTAGGCATTCTGGCTCAC	5′-TCCCACTGTTCCATATCGTCA	519	*Escherichia coli* pAT121	Giovanetti *et al.* (2003)
tet(S)	5′-GGAGTACAGTCACAAACTCG	5′-GGATATAAGGAGCAACTTTG	335	*E. coli* pVP2	A. van Hoek, personal communication
tet(W)	5′-GAGAGCCTGCTATATGCCAGC	5′-GGGCGTATCCACAATGTTAAC	168	*Lactobacillus reuteri* ATCC 55730	Vancraeynest *et al.* (2004)
erm(A)	5′-AAGCGGTAAACCCCTCTGAG	5′-TCAAAGCCTGTCGGAATTGG	441	*Staph. aureus* 694/01	Jensen *et al.* (1999)
erm(B)	5′-CATTTAACGACGAAACTGGC	5′-GGAACATCTGTGGTATGGCG	425	*Enterococcus faecalis* TUH a1-19	Jensen *et al.* (1999)
erm(C)	5′-ATCTTTGAAATCGGCTCAGG	5′-CAAACCCGTATTCCACGATT	295	*Staph. aureus* 694/01	Jensen *et al.* (1999)
erm(T)	5′-TATTATTGAGATTGGTTCAGGG	5′-GGATGAAAGTATTCTCTAGGGATTT	395	*Lact. reuteri* 100-63	Tannock *et al.* (1994)
mef(A)	5′-AGTATCATTAATCACTAGTGC	5′-TTCTTCTGGTACTAAAAGTGG	348	*Streptococcus pyogenes*	Giovanetti *et al.* (2003)
cat-TC	5′-CAATAGCGACGGAGAGTTAGG	5′-AATCCTGCATGATAACCATCAC	384	–	This study
16S rDNA	5′-AGAGTTTGATCCTGGCTC	5′-CGGGAACGTATTCACCG	1400	–	Egervärn *et al.* (2007a)

*Positive control strains were kindly provided by Wolfgang Witte (*Staph. aureus* 1880/04, *Staph. aureus* 694/01), Morten Danielsen (*Ent. faecium* 5878, *Lact. plantarum* 5057), Askild Holck (*E. coli* pAT121, *E. coli* pVP2, *Ent. faecalis* TUH a1-19), BioGaia AB (*Lact. reuteri* ATCC 55730), Gerald Tannock (*Lact. reuteri* 100-63), Eleonora Giovanetti (*Strep. pyogenes*).

Supercoiled DNA ladder (2–10 kb; Promega Biosciences, San Luis Obispo, CA, USA) was used to estimate the molecular mass of the plasmids. For strain F14, the single DNA band observed was cut out from the agarose gel, and the DNA was purified using Qiaex II Gel extraction kit (Qiagen).

Southern blot

Plasmid DNA was transferred by blotting (VacuGene XL; Pharmacia Biotech, Uppsala, Sweden) to nylon filters (Hybond-N+; GE Healthcare, Buckinghamshire, UK) and hybridized with [α-^{32}P]dCTP-labelled DNA probes. Total DNA from each strain was spotted at the bottom of the filter as a positive control. The *tet*(M) and *tet*(W) probes used were PCR products obtained with the forward primers as reported in Table 1 and the following reverse primers 5′-CTCTGTTCAGGTTTACTCGG-3′ for *tet*(M) and 5′-GTCCTCACGCCACCTTTTACG-3′ for *tet*(W). Approximately 50 ng of total DNA from *Lact. plantarum* E-042709 and *Lact. reuteri* ATCC 55730 was used as template, generating a *tet*(M)-amplicon of 853 bp and a *tet*(W)-amplicon of 963 bp respectively. Labelling of probes was performed using ready-to-go DNA labelling beads (dCTP; GE Healthcare) and Redivue dCTP 5′-[α-^{32}P]-triphosphate (GE Healthcare). Filter hybridization was performed according to standard methods, and hybridization images were obtained with a phosphor imager (Personal Molecular Imager FX; Bio-Rad, Hercules, CA, USA) and analysed with Quantity One V4.6.2 software (Bio-Rad).

Determination of gene location by real-time PCR

The location of the antibiotic resistance genes was also determined by comparing the cycle threshold (C_t) between reactions with template from a plasmid DNA and from a total DNA extraction. The principle is that a plasmid-located gene is more common in a plasmid DNA preparation than in a total DNA preparation, and contrary to this, a chromosome-located gene is more common in a total DNA preparation than in a plasmid DNA preparation. The ΔC_t obtained for each resistance gene was normalized by the ΔC_t of the reference chromosomal gene, *16S rDNA*, in order to level out the differences in DNA quality between the DNA preparations. The $\Delta\Delta C_t$ was calculated according to:

$$\Delta\Delta C_t = \Delta C_t(\text{16S rDNA gene}) - \Delta C_t(\text{resistance gene})$$

where $\Delta C_t = C_t$ (plasmid DNA)$-C_t$ (total DNA).

The conditions for gene amplification were the same as those described earlier with the exception that 1 ng of template DNA was used in each reaction and the

annealing temperature for 16S rDNA was set to 49°C. Primers used were reported in Table 1.

Determination of flanking regions

Total DNA was extracted as described previously and concentrated using isopropanol precipitation according to standard methods. Flanking regions of resistance genes were sequenced by using genomic DNA as template and primers directed outwards from the PCR products of the detected resistance genes. Subsequent primers were designed to anneal to the outer end of the sequences obtained. Sequencing was performed according to the BigDye Terminator ver. 3.1 Cycle Sequencing kit for large DNA templates (Applied Biosystems) with slight modifications. In brief, the PCR mixture was prepared in a 20-μl volume containing 8·0 μl Terminator Ready Reaction mix, 2 μg total DNA and 3·2 pmol primer. The cycle sequencing reaction was performed, using GeneAmp PCR System 2700 with the following conditions: 5 min at 95°C, 90 cycles with 30 s at 95°C, 10 s at 52°C and 4 min at 60°C. The samples were purified using Centri-Sep spin columns (Applied Biosystems) prior to loading onto an ABI3700 capillary instrument. Sequencing was performed at the Uppsala Genome Center. The sequences obtained flanking the resistance genes were assembled using Contig Express in Vector NTI software (Invitrogen, Carlsbad, CA, USA). The complete sequences were analysed with Artemis ver. 8 software (The Sanger Institute, Cambridge, UK), and database searches of identified ORFs were performed using Blast via the GenBank database. The genome sequence of *Lact. reuteri* strain ATCC 55730 (Båth *et al.* 2005) containing the plasmid pLR581 with Genbank accession number EU583804 (Rosander *et al.* 2008) was used for analysing the flanking regions of *tet*(W) in that strain.

Nucleotide sequence accession number

The nucleotide sequences of *tet*(W) and *erm*(C) and their flanking regions of strain PA-16 have been submitted to the GenBank database under accession number FJ489649 and FJ489650 respectively.

Results

Identification of resistance genes

All 32 *Lact. reuteri* strains and two *Lact. plantarum* strains previously reported with atypical MICs for tetracycline and/or erythromycin were screened for the presence of known resistance genes. Real-time PCR revealed the presence of *tet*(W) in 24 of the 28 *Lact. reuteri* strains with atypical MIC (>64 μg ml^{-1}) for tetracycline (Fig. 1), whereas *tet*(M) was detected in both *Lact. plantarum* strains. None of the other five tetracycline resistance genes tested were found in any strain including the four *tet*(W) negative strains, Cow 10, ATCC 55148, MF2-3 and MF14-C. In addition, a tetracycline resistance gene was not detected in the strains MF2-3 and MF14-C when tested by a second screening using a microarray assay detecting 33 different oxy/tetracycline resistance genes.

Four of the six *Lact. reuteri* strains with atypical MIC (>256 μg ml^{-1}) for erythromycin were positive for *erm*(B), and one strain each was positive for *erm*(C) and *erm*(T) (Fig. 1). All strains were negative for the other four erythromycin resistance genes tested. One (LMG 18391) of the two *Lact. reuteri* strains displaying atypical MIC for both tetracycline and erythromycin carried *tet*(W) and *erm*(B), whereas the second strain (PA-16) carried *tet*(W) and *erm*(C). The six strains (100-67, 8557:1, LpA1, 1068, BR11 and ATCC PTA5289) that displayed low MICs for tetracycline and/or erythromycin, but were genetically similar to strains with atypical MICs for these antibiotics, were all negative in the PCR screening for resistance genes (Fig. 1). Strain 5010, with a chloramphenicol MIC of 128 μg ml^{-1} (Egervärn *et al.* 2007b) and a typical tetracycline MIC of 16 μg ml^{-1}, harboured a *cat*-TC gene and a *tet*(W) gene, as determined with real-time PCR and a microarray assay (Fig. 1). Strain ATCC PTA6127, displaying a rep-PCR fingerprint similar to strain 5010 and isolated from dog, had typical tetracycline and chloramphenicol MICs. This strain was negative for the tetracycline and chloramphenicol genes tested. Screening results were confirmed by sequencing of a PCR amplicon for each type of resistance gene (data not shown).

Location of identified resistance genes

Plasmid DNA preparations from seven *tet*(*W*) positive *Lact. reuteri* strains and one *tet*(M) positive *Lact. plantarum* strain were subjected to Southern blot to determine whether the resistance genes were located on plasmid. A *tet*(*W*)-positive signal was detected for four of the strains tested (Fig. 2a). Three of these strains, having highly similar rep-PCR fingerprints (shaded fields, Fig. 1), displayed the same plasmid profile and the same band of approx. 12 kb hybridized to the *tet*(W) probe (Fig. 2a). For the *Lact. plantarum* strain tested, the *tet*(M) probe hybridized to a band with an estimated size of 11 kb (Fig. 2b).

Gene location was also determined by real-time PCR, calculating the difference in C_t between reactions with template from a plasmid DNA and from a total DNA extraction respectively (Fig. 1). Comparison of $\Delta\Delta C_t$ values and Southern blot data for the eight strains

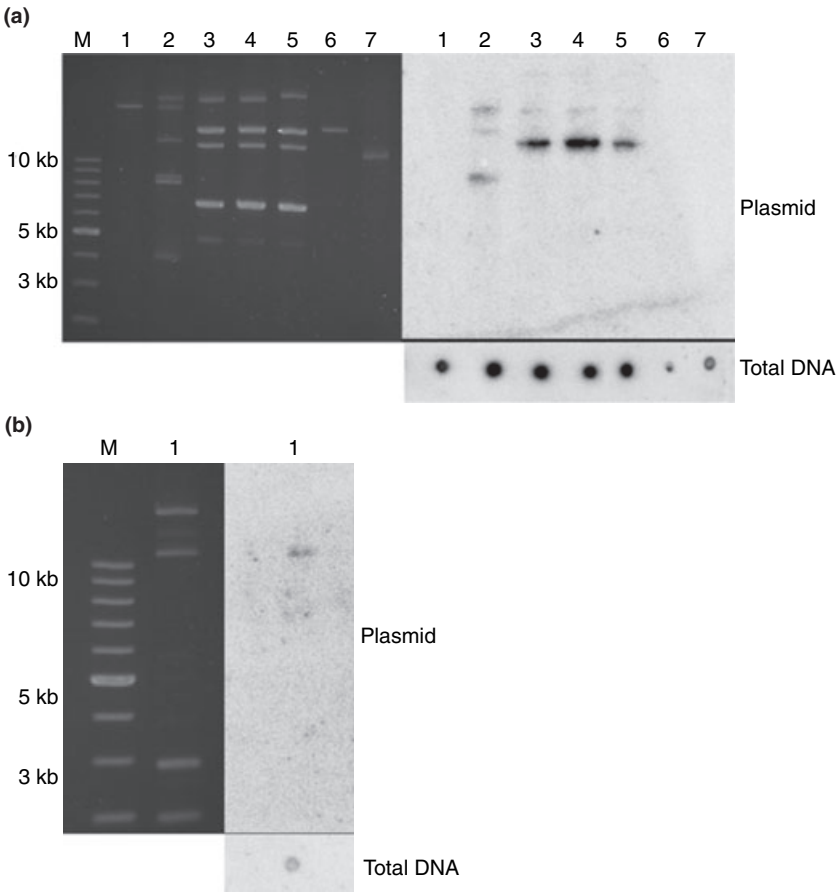


Figure 2 Southern blots of plasmid DNA preparations from seven *tet*(W)-positive *Lactobacillus reuteri* strains (a) and one of the *tet*(M)-positive *Lactobacillus plantarum* strains (b). 0·8% agarose gels stained with ethidium bromide and the corresponding Southern blots hybridized with the *tet*(W) (a) and the *tet*(M) (b) probe respectively. (a) Lanes: M, ccc-DNA size standard; 1, PA-16; 2, LMG 18391; 3, ATCC 55730; 4, MV6-2A; 5, CF48-3A1; 6, DSM 20015; 7, 3040. (b) Lanes: M, ccc-DNA size standard; 1, VTT E-042708. Total DNA preparations were included as positive controls for strains carrying the resistance gene on the chromosome.

tested with both methods revealed that $\Delta\Delta C_t$ value was above 5·9 for the five strains carrying *tet*(W) or *tet*(M) on a plasmid and between 0 and 1 for the three strains carrying the resistance genes on the chromosome (Fig. 3). Applying the real-time PCR method on additional 17 *cat*-, *erm*- and/or *tet*-positive strains resulted in $\Delta\Delta C_t$ values above 3·7 or below 1 (Fig. 3). For the

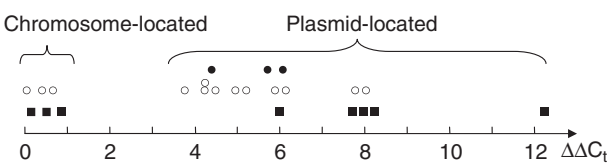


Figure 3 Schematic presentation of $\Delta\Delta C_t$ values obtained by real-time PCR and used for determination of gene location in *cat*-, *tet*- and/or *erm*-positive strains. Values of $\Delta\Delta C_t < 1$ indicate chromosome-located resistance genes and above 3·7 plasmid-located genes. (○) Location determined with real-time PCR only; (■) location confirmed with Southern hybridization and (●) location previously reported as plasmid encoded (S.E. Ahrné, personal communication; Axelsson *et al.* 1988). The negative $\Delta\Delta C_t$ value of the plasmid-free strain, 2010, is shown in Fig. 1.

tet(W) positive strains tested with the real-time PCR method only, $\Delta\Delta C_t$ values above 4·1 and between 0 and 1 were obtained for five and three strains respectively, whereas strain 2010 without plasmids (data not shown) had a negative $\Delta\Delta C_t$ value (Fig. 1). The $\Delta\Delta C_t$ value of the *tet*(W)-positive strain F14 was increased from 2·2 to 4·2 by first purifying the DNA of its single plasmid (data not shown). All *tet*(W)-positive strains within the group with highly similar rep-PCR fingerprints displayed a $\Delta\Delta C_t$ value of above 5·0 (shaded fields, Fig. 1). The second *Lact. plantarum* strain, with a $\Delta\Delta C_t$ value of 8·0, had an identical plasmid profile to the *Lact. plantarum* strain subjected to Southern blot, which had a $\Delta\Delta C_t$ value of 12·2 (data not shown).

The $\Delta\Delta C_t$ value was above 3·7 for the six *erm* positive *Lact. reuteri* strains (Fig. 1). All these strains, which were clustered in the dendrogram (Fig. 1), had different plasmid profiles, with the number of bands ranging from one to six (Fig. 4). All *erm* genes, except for *erm*(B) in strains 8557:1 and 1068, correlated to bands of different sizes (Fig. 4), as determined by Southern blot (data not shown) or reported previously (S.E. Ahrné, personal communication; Axelsson *et al.* 1988; Tannock *et al.* 1994).

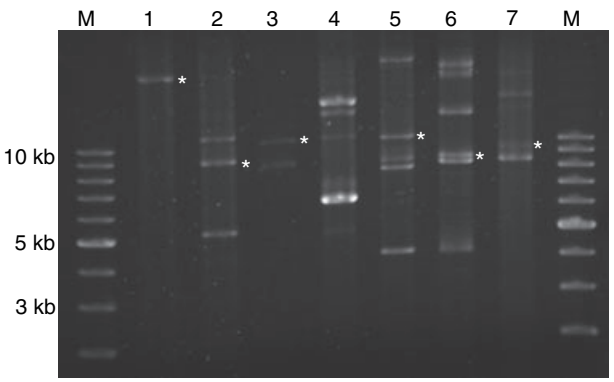


Figure 4 Plasmid profiles of the six *erm*-positive *Lactobacillus reuteri* strains and one erythromycin susceptible *Lact. reuteri* strain isolated from pig. 0·8% agarose gel stained with ethidium bromide. Lanes: M, ccc-DNA size standard; 1, PA-16 – *erm*(C); 2,100-67 – *erm*(T); 3, 8557:1 – *erm*(B); 4, LpA1 – susceptible; 5, 1068 – *erm*(B); 6, LMG 18391 – *erm*(B); 7, 1048 – *erm*(B). The asterisks indicate the position of the *erm* genes, determined by Southern blot (data not shown) or reported previously (S.E. Ahrné, personal communication; Axelsson *et al.* 1988; Tannock *et al.* 1994).

Flanking regions of identified resistance genes

Direct genome sequencing was used to determine upstream and downstream sequences of the PCR products of the plasmid-bound *erm*(C) and chromosomally located *tet*(W) genes identified in *Lact. reuteri* strain PA-16. The DNA sequences obtained, together with the plasmid-bound *tet*(W) gene of *Lact. reuteri* strain ATCC 55730, are schematically presented in Fig. 5. The sequences of the two *tet*(W) genes were almost identical. Several genes related to arsenic resistance were present upstream of *tet*(W) in ATCC 55730, whereas two hypothetical proteins and a protein involved in Mg^{2+} transportation were present in PA-16. Both *tet*(W) genes were preceded by a putative *tet*(W) regulatory peptide showing 100% similarity to corresponding ORFs in *tet*(W)-positive bifidobacterial strains (B. Mayo, personal communication). The 3′ flanking sequences of the *tet*(W) genes contained various elements such as an integrase in ATCC 55730 and a transposase in PA-16.

The deduced amino acid sequence of *erm*(C) showed 99%, 98% and 95% similarity with the *erm*(C) gene in a *Staphylococcus hyicus*, *Staphylococcus saprophyticus* and *Staphylococcus aureus* isolate respectively. A plasmid replication protein (Rep) and a hypothetical protein were located upstream of the *erm*(C) gene, whereas two ORFs coding for two different transposases flanked the downstream region (Fig. 5).

Discussion

There are currently 41 oxy/tetracycline resistance genes described, of which *tet*(K, L, M, O, Q, S, W, 36) have been reported in various *Lactobacillus* species (Chopra and Roberts 2001; Roberts 2005; Ammor *et al.* 2008; Brown *et al.* 2008). Our study found that the two *Lact. plantarum* strains with atypical MIC for tetracycline were positive for *tet*(M), which is the most widely distributed *tet* gene in general (Roberts 2005), including lactobacilli. The gene mediates ribosomal protection against tetracycline and has previously been identified in the strains of *Lact. plantarum*, *Lactobacillus alimentarius*, *Lactobacillus curvatus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus crispatus* and *Lactobacillus sakei* (Gevers *et al.* 2003a; Klare *et al.* 2007). The *tet*(M) and *tet*(S) are the only *tet* genes found so far in *Lact. plantarum* (Danielsen 2002; Gevers *et al.* 2003a; Huys *et al.* 2006). When localized in this species, *tet*(M) has been found on a plasmid with a size of approx. 10 kb (Danielsen 2002; Gevers *et al.* 2003a), which was also the case in our study.

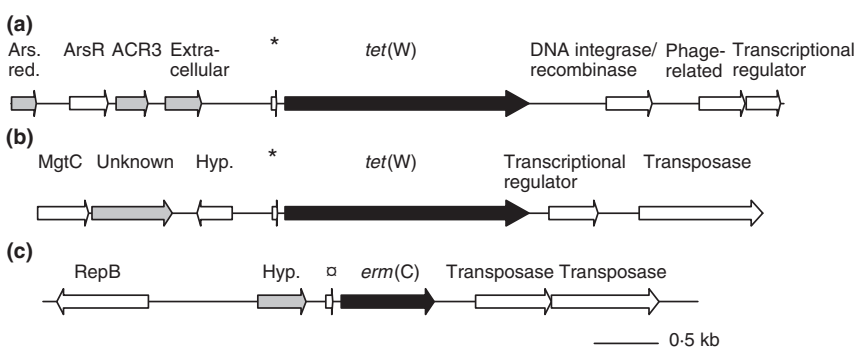


Figure 5 Schematic presentation of resistance genes and flanking regions in *Lactobacillus reuteri*: (a) *tet*(W) of ATCC 55730; (b) *tet*(W) of PA-16; (c) *erm*(C) of PA-16. Grey arrows indicate pseudogenes. Hyp., hypothetical protein; Ars. red., arsenite reductase; ArsR, arsenite transcriptional regulator; ACR3, arsenite efflux pump; MgtC, MgtC/SapB transporter; RepB, replication initiation protein; **Tet*(W)-regulatory peptide; ¤*Erm*(C) leader peptide. (b) The sequences in the 5′-end of MgtC and in the 3′-end of the transposase, 83/216 and 20/343 amino acids respectively, are missing.

The *tet*(W) gene, also encoding a protein protecting the ribosome from tetracycline, is commonly found in human and animal intestinal bacteria, such as various species of *Bifidobacterium*, *Butyrivibrio*, *Mitsuokella* and *Roseburia* (Kazimierczak *et al.* 2006). However, it appears to be less widely distributed in lactobacilli and has so far only been reported in few strains of *Lact. crispatus*, *Lactobacillus johnsonii*, *Lactobacillus paracasei* and *Lact. reuteri* (Kastner *et al.* 2006; Klare *et al.* 2007; Huys *et al.* 2008). The *Lact. reuteri* strains included here were a subset of the 56 strains previously assessed for antibiotic susceptibility (Egervärn *et al.* 2007a). The strains were selected to provide a wide distribution in terms of genetic diversity, source, temporal and spatial origin. The presence of the *tet*(W) gene in 24 (86%) of the 28 strains with atypical MIC for tetracycline shows that *Lact. reuteri*, displaying 40–42% G+C content (Hammes and Hertel 2006), is frequently associated with *tet*(W). In contrast, the closely related species *Lactobacillus fermentum*, which has a higher G+C content of 52–54% (Hammes and Hertel 2006), is susceptible to tetracycline, as shown previously by Egervärn *et al.* (2007a). Interestingly, this is in contrast to the proposed theory that *tet*(W), which has a much higher G+C content (53%) than other ribosome-protection-type *tet* genes, would be associated with bacterial hosts with a similar G+C-content, such as bifidobacteria (Scott *et al.* 2000). Whether the widespread presence of *tet*(W) in genetically diverse *Lact. reuteri* strains is because of a repeated uptake of the gene or that a common ancestor became *tet*(W) positive and that some strains have lost their *tet*(W) gene over time is an open question. An argument for the former hypothesis is that *tet*(W) has been found in many species present in the gastrointestinal tract of both humans and animals (Scott *et al.* 2000) and is often associated with conjugative transposons (Roberts 2005). Differences with respect to flanking regions of the two sequenced *tet*(W) genes would also suggest multiple independent acquisitions. However, although sequence analysis of the 12-kb-plasmid harbouring *tet*(W) in *Lact. reuteri* ATCC 55730 revealed a downstream integrase, no known origin of transfer, nor any described *tra* or *mob* genes were found.

In this study, the results of the PCR and microarray screening correlated well with MIC data reported earlier (Flórez *et al.* 2006; Egervärn *et al.* 2007a), except for strain 5010 that was *tet*(W) positive in the PCR screening but displayed a tetracycline MIC of 16 μg ml^{-1}. However, a weaker hybridization signal for the *tet*(W) oligonucleotide was observed on the microarray for strain 5010 compared with control strains, indicating the presence of a partial or mutated *tet*(W) gene, or a gene that is similar, rather than identical to, *tet*(W). Furthermore, there were two genetically closely related tetracycline-resistant strains that were negative in the PCR screening and to the 33 *tet* genes tested in the microarray assay. The two strains differed regarding resistance phenotype by not displaying a high level resistance to tetracycline until after 48-h incubation, whereas the other strains had MICs ≥128 μg ml^{-1} already after 24 h (data not shown). Thus, the two strains either carried one of the recently identified *tet* genes, a novel tetracycline resistance gene, or the high MIC with time could be because of a multidrug efflux pump removing tetracycline from its target. Taken together, this shows that both phenotypic and genetic methods are needed to guarantee the presence or absence of acquired resistance genes in strains intended for use in food, feed and probiotic applications, as has been pointed out previously (Danielsen 2002; Hummel *et al.* 2007).

The clear grouping of the $\Delta\Delta C_t$ values, below 1 for chromosomal location and above 3·7 for plasmid location (Fig. 3), and the agreement between the $\Delta\Delta C_t$ values and Southern blot data, as well as to the location previously determined for three plasmid-encoded resistance genes (S.E. Ahrné, personal communication; Axelsson *et al.* 1988), show that the real-time PCR method developed here could be used for determining whether a gene is plasmid located or not. However, plasmids larger than 50 kb are not efficiently isolated with the Qiagen kit used, and thus, resistance genes situated on such large plasmids appear to be chromosomally encoded.

Lactobacilli are generally susceptible to chloramphenicol, but different *cat* genes, encoding a chloramphenicol acetyltransferase, have previously been found on a plasmid in an *Lact. plantarum* strain isolated from pork (Ahn *et al.* 1992) and in an *Lact. reuteri* strain from chicken (Lin *et al.* 1996). The *Lact. reuteri* dog strain 5010 with an atypical chloramphenicol-MIC tested positive in the *cat*-TC-specific PCR, using primers based on the known *Lact. reuteri cat* gene (Lin *et al.* 1996). Resistance to both erythromycin and clindamycin is commonly attributed to *erm* genes. The 33 *erm* genes found so far encode an rRNA methylase, which methylates the binding site in the 50S ribosomal subunit, the overlapping target for macrolide, lincosamide, streptogramin B (MLS_B) and ketolide antibiotics (Roberts 2008). MLS_B resistance in lactobacilli is frequently associated with the presence of *erm*(B) and in a few cases with *erm*(C), *erm*(G) and *erm*(T) (Roberts 2003; Ammor *et al.* 2007). In our study, a plasmid-encoded *erm*(B) gene was detected in four of the six *Lact. reuteri* strains with atypical MICs to erythromycin and clindamycin, which was in agreement with previous studies on three of these strains (1048, 1068, 8557:1) (S.E. Ahrné, personal communication; Axelsson *et al.* 1988). The presence of both *erm*(B) and *tet*(W) in an *Lact. reuteri* strain (LMG 18391) is a novel finding, although this gene combination was recently found in an *Lact. paracasei* strain

(Huys *et al.* 2008) and two *Lact. crispatus* strains (Klare *et al.* 2007). We also found that the two genes were located on the same plasmid as determined by Southern blot [data not shown for *erm*(B)]. However, it remains to be determined whether the genes are linked to a conjugative transposon, which is often the case with linked *erm*(B) and *tet*(M) (Roberts *et al.* 1999).

To our knowledge, this is the first study reporting the presence of an *erm*(C) gene in *Lact. reuteri*. The nucleotide sequence of *erm*(C) in strain PA-16 revealed an rRNA methylase gene with high similarity (95–99% amino acid identity) to *erm*(C) genes present in various *Staphylococcus* species. The gene is in both cases located on a plasmid, usually small plasmids (<5 kb) in staphylococci, whereas the plasmid size in PA-16 was estimated to approx. 20 kb. The upstream region of the *erm*(C) gene contained a plasmid Rep also found in other *Lactobacillus* plasmids, verifying that the resistance gene with a $\Delta\Delta C_t$ value of 3·8 was plasmid encoded. The transposases located downstream of the *erm*(C) gene and the chromosomally located *tet*(W) gene of the same strain may be part of transfer machineries, facilitating the spread to other strains.

The macrolide tylosin was the most commonly used antimicrobial agent in pig farming in the European Union until it was banned as an animal growth promoter in 1999. Today, it is still used for therapeutic purposes (A. Franklin, personal communication). Consequently, bacteria such as enterococci and staphylococci isolated from pigs are frequently resistant to macrolides (Aarestrup and Carstensen 1998). Here, we found that four of the six *Lact. reuteri* strains positive for an *erm* gene were originally isolated from pig, although only six of the 56 strains were from this source. The unique plasmid profiles of the *erm*-positive strains imply that the erythromycin resistance was not spread clonally, but have rather been taken up at separate events.

In this study, genetic characterization of 38 strains of *Lact. reuteri* from various human and animal habitats and *Lact. plantarum* isolated from silage revealed that *tet*(W) and various *erm* genes were found in almost all *Lact. reuteri* strains with atypical MICs for tetracycline and/or erythromycin and thus appear to be the most common resistance determinants within this species. The *erm*(C) gene found in one of the *Lact. reuteri* strains was a novel finding, whereas the *tet*(M) gene identified in both *Lact. plantarum* strains was more expected. Most resistance genes were located on plasmids, but although this study showed that the plasmid harbouring *tet*(W) in *Lact. reuteri* strain ATCC 55730 seems to be nonconjugative and nonmobilizable and although a previous filter-mating experiment by Kastner *et al.* (2006) failed to show transferability of the *tet*(W) gene, the transfer ability cannot be ruled out. A recent report on *in vitro* transfer of an *erm*(B) gene in an *Lact. reuteri* strain from an African pig to *Enterococcus faecalis* showed that this could indeed happen in this species (Ouoba *et al.* 2008). In food-related *Lact. plantarum* strains too, interspecies horizontal transfer of *tet*(M) and *erm*(B) genes has been demonstrated *in vitro* (Gevers *et al.* 2003b) and in the GIT of germ-free rats (Jacobsen *et al.* 2007). Currently, there is a discussion about the risk with antibiotic resistance genes present in strains used commercially. Careful selection and screening processes have to be undertaken before applying an environmentally isolated *Lactobacillus* strain as a new starter culture or a probiotic product. The here presented data will be used in future risk assessments of the two widely used species *Lact. reuteri* or *Lact. plantarum*.

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