Antibiotic resistance of *Enterococcus* strains isolated from rabbit faeces and molecular characterization of the plasmid pCMT1 harboured by *Enterococcus faecium* 8G

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Introduction

Rabbits are monogastric herbivores, which are widely used for research purposes and husbandry. Digestive disorders are the predominant cause of mortality in commercial rabbits. These diseases have been usually treated by administration of antimicrobial substances. Nevertheless, there is an increasing concern on the preventive use of antibiotics in animal feeding and probiotics may constitute a safe alternative. In a previous study, the facultative anaerobic intestinal microbiota of healthy rabbits was characterised for the selection of potential probiotic strains (Linaje et al. 2004). Enterococci were the only lactic acid bacteria isolated from rabbit faecal samples. Although certain strains of *E. faecalis* and *E. faecium* have been associated with food-borne illnesses and nosocomial infections, several enterococci are commonly used as starter cultures of different cheeses and milk fermented products. Furthermore, probiotic properties have been claimed for certain enterococci.

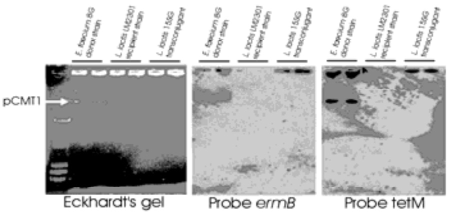
The main concern for a safe use of enterococcal strains is their possible role as vehicles for the transference of antibiotic resistance genes to pathogenic bacteria. Therefore, the antibiotic resistance of selected enterococcal isolates was studied within our scheme of selection of probiotic strains for rabbits.

Conjugal transfer of antibiotic resistant markers from *E. faecium* 8G to *Lactococcus lactis* LM2301

Resistance to Clo, Ery and Tet was always simultaneously transferred from *E. faecium* 8G to *L. lactis* LM2301, however at a low frequency (<10⁻⁷ transconjugants/donor).
Southern analyses confirmed the presence of genes *catA*, *ermB* and *tetM* in *L. lactis* transconjugants.
Phenotypic analyses (API 50CHS) did not show any additional differences between the receptor strains and transconjugants.
L. lactis transconjugants were unable to transfer antibiotic resistance to *E. faecalis* 4C under our experimental conditions.

Resistance genes are harboured by a plasmid

Electrophoretic analysis by using a modification of the Eckhardt's procedure revealed the presence of two large plasmids in *E. faecium* 8G.
Southern analyses showed that genes *catA*, *ermB* and *tetM* are harboured by the smaller plasmid (pCMT1).
Plasmid pCMT1 was not detected in L. lactis transconjugants. However, probes specifically hybridized with material that did not enter the gel (chromosomal DNA). This result suggests that pCMT1 integrates in the chromosome of *L. lactis* LM2301.

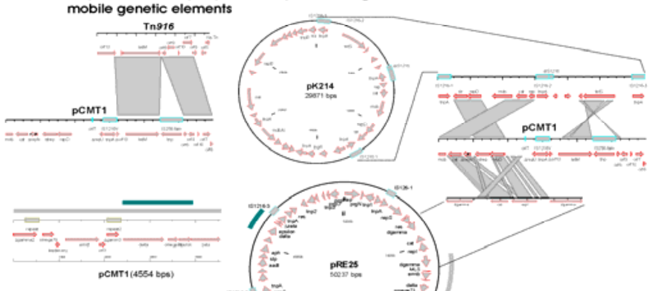


Electrophoretic analysis by a modified Eckhardt's procedure and Southern hybridization of cell lysates of *E. faecium* 8G, *L. lactis* LM2301 and the *L. lactis* transconjugant isolate 15SG.

Partial sequencing of pCMT1 and comparative analysis

Regions of pCMT1 around genes *ermB* and *tetM* were sequenced by inverse PCR (4554 and 11958 bp, respectively).
Region around *ermB* is virtually identical to that present in its counterparts in pIP501, pRE25 and other related antibiotic resistance plasmids.
The analysis of the region around *tetM* reveals a composite structure: An *IS1256* element separates two distinct regions:
Region I spans gene *tetM* and downstream sequence. It is homologous to conjugative transposons like *Tn916* excepting for the insertion of an *IS256* element in pCMT1.
Region II is constituted by a number of modules also present in other plasmids; their main characteristics and similar sequences are indicated in the accompanying Tables.
Some of these modules are limited by direct repeats, suggesting that, in addition to IS elements, homologous recombination among direct repeats has played a major role in the assembly of pCMT1 as previously observed for other related plasmids (Zúñiga et al. 2003).

Sequence similarity of pCMT1 sequenced fragments with other characterized mobile genetic elements



Antibiotic resistance of *Enterococcus* strains isolated from rabbits

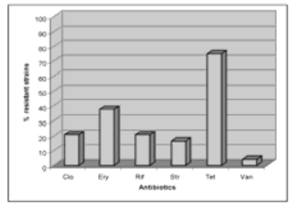
All strains were resistant to kanamycin and sensitive to ampicillin, fusidic acid and gentamycin. 18 out of 24 strains were resistant to Tet. Gene *tetM* was detected in all tet resistant strains. Gene *ermB* was detected in all 9 ery resistant strains. 3 out of 5 clo resistant strains harboured gene *catA*. Only *E. gallinarum* 10C was moderately resistant to Van.

Phenotype	Antibiotic resistance[1]	Structural antibiotic resistance genes[2]
E. durans		
2F	Tet R, Kan R, Rif R	*tetM*
E. gallinarum		
10C	Van R, Kan R	
E. faecalis		
1A	Tet R, Kan R	*tetM*
2E	Tet R, Kan R	*tetM*
3A	Ery R, Tet R, Kan R	*tetM, ermB*
3B	Tet R, Kan R	*tetM*
4A	Kan R	
4C	Kan R	
6B	Kan R	
7A	Ery R, Str R, Tet R, Kan R	*tetM, ermB*
6C	Ery R, Tet R, Clo R-, Kan R	*tetM, ermB*
7B	Ery R, Str R, Tet R, Kan R	*tetM, ermB*
7C	Ery R, Str R, Tet R Clo R-, Kan R	*tetM, ermB*
7D	Ery R, Str R, Tet R, Kan R	*tetM, ermB*
8A	Tet R, Kan R, Rif R-	*tetM*
8F	Tet R, Kan R	*tetM*
E. faecium		
2A	Tet R, Kan R	*tetM*
2C	Kan R	
3C	Tet R, Kan R, Rif R	*tetM*
3D	Kan R, Rif R	
8B	Ery R, Tet R, Clo R, Kan R	*tetM, ermB, catA*
8C	Ery R, Tet R, Clo R, Kan R	*tetM, ermB, catA*
8D	Tet R, Kan R, Rif R-	*tetM*
8G	Ery R, Clo R, Tet R, Kan R	*tetM, ermB, catA*

1 Antibiotic resistance was determined on agar plates supplemented with the appropriate antibiotic. Clo, chloramphenicol; Ery, erythromycin; Kan, kanamycin; Rif, rifampicin; Str, Streptomycin; Tet, tetracyclin.
2 Antibiotic resistance genes were detected by PCR

Primers used for PCR detection of antibiotic resistance genes

Antibiotics	Model genetic element	Acc. No	Gene	Oligonucleotides: Name	Oligonucleotides: Sequence
Chloramphenicol	pIP501	X65462	*catA* *	catA-f	5'-GGGATAGAAAAGAATATTTTG-3'
		*		catA-r	5'-GAACTAAAATCAATCCAAGG-3'
	pC194	NC_002013	*catB*	catB-f	5'-CAATTGGAAGAGAAAAGAG-3'
				catB-r	5'-CCTGCATGATAACCATCAC-3'
	pACYC184	X06403	*catC* *	catC-f	5'-AATCACTGGATATACCACCG-3'
				catC-r	5'-AAGCATTCTGCCGACATG-3'
Erythromycin	pIP501	X72021	*ermB*	ermB-f	5'-AAATTGGAACAGGTAAAGGGC-3'
				ermB-r	5'-CAAGCAATGAAACACGCCAAAG-3'
	ND	AY071835	*mefA*	mefA-f	5'-TATAGTCAGTCTTTGCAGTC-3'
				mefA-r	5'-CACGTCCTAAATATTCAGG-3'
Tetracyclin	Tn916	X56353	*tetM*	tetM-f	5'-GAGTTTTAGCTCATGTTGATGC-3'
				tetM-r	5'-TGCTTTCCTCTTGTTCAGTTC-3'



Conclusions

Resistance to tetracycline, and to a lesser extent erythromycin, is common among *Enterococcus* strains isolated from rabbits.

Enterococcus faecium 8G harbours plasmid pCMT1 which encodes resistance at least to chloramphenicol, erythromycin, streptomycin and tetracycline , and it can be transferred by conjugation to *L. lactis*.

Plasmid pCMT1 has a mosaic structure: the comparative analysis suggests that region I is the result of several integration events of a number of rolling circle antibiotic resistance plasmids and regions almost identical to their counterparts in other enterococcal mobile genetic elements.

The phylogenetic analyses of *cat and str* shows a close relatedness of pCMT1 genes to their counterparts in pK214 and some staphylococcal plasmids. Therefore, pCMT1 has acquired a number of genetic modules not found in other enterococcal plasmids previously described.

In summary, the structural differences observed and the phylogenetic analyses suggest that the assembly of pCMT1 occurred independently of the assembly of other antibiotic resistance plasmids found in enterococci.

Charactistics of genes located in region I

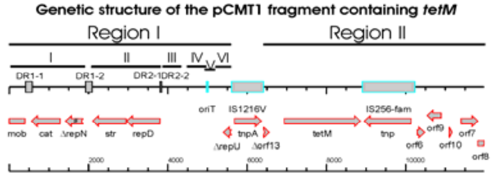
Gene	Domains	Function	Observations
mob	pfam01076, Mob_Pre, Plasmid recombination enzyme	Conjugative mobilisation	Uncompleted sequence
cat	pfam00302, CAT, Chloramphenicol acetyltransferase	Chloramphenicol resistance	
ΔrepN	pfam02486, Rep_trans, Replication initiation factor	Initiation of DNA rolling circle replication	Non functional
str	pfam04439, Adenyl_transf, Streptomycin adenylyltransferase	Aminoglycoside antibiotics resistance	
repD	pfam02486, Rep_trans, replication protein	Initiation of DNA rolling circle replication	
oriT		Origin of transference (mobilization)	Pos. 4970-5011
ΔrepU	pfam01446, Rep_1, Replication protein	Initiation of DNA rolling circle replication	Non functional

Analysis of modules detected in pCMT1 region I

Modules	Coordinates	BlastN best hits[1]		Organism	Relevant features
I	1-1851	Plasmid delta pWDB100 [412-554 (93%)] [560-1851 (98%)]	X68412	*Listeria monocytogenes*	*mob, cat, ΔrepN*[2]
		Plasmid pSBK203 [430-554 (95%)] [570-1851 (99%)]	U35036	*Staphylococcus aureus*	
		Plasmid pK214 [1-554 (90%)] [570-1848 (98%)]	X92946	*Lactococcus lactis*	
II	1951-3821	Plasmid pK214 [1951-3821 (98%)]	X92946	*Lactococcus lactis*	*str, repD*
		Unnamed plasmid [2216-3821 (99%)][3]	AY939911	*Enterococcus casseliflavus*	
		Plasmid pS194 [1951-3059 (97%)] [3178-3710 (89%)]	SAPS194	*Staphylococcus aureus*	
III	3833-4398	Plasmid delta pWDB100 [3833-4396 (92%)]	X68412	*Listeria monocytogenes*	Noncoding region
		Plasmid pRE25 [3833-4024 (90%)] [4098-4383 (91%)]	X92945	*Enterococcus faecalis*	
		Unnamed plasmid [3835-4042 (89%)] [4098-4398 (90%)]	AY939911	*Enterococcus casseliflavus*	
IV	4523-4995	Plasmid pRE25 [4523-4995 (98%)]	X92945	*Enterococcus faecalis*	Noncoding region
		Plasmid delta pWDB100 [4692-4995 (95%)]	X68412	*Listeria monocytogenes*	
		Unnamed plasmid [4556-4770 (97%)]	AY939911	*Enterococcus casseliflavus*	
V	4956-5190	Plasmid pBF1 [4964-5190 (96%)]	AJ716330	*Lactobacillus plantarum*	*oriT*
		Plasmid pA1 [4956-5190 (93%)]	Z11717	*Lactobacillus plantarum*	
		Plasmid p121BS [4966-5190 (92%)]	AF310974	*Lactobacillus* sp. strain PC121B	
VI	5191-5597	No significant hits			*ΔrepU*[4]

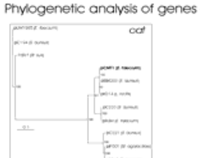
[1]Only major hits have been indicated, coordinates correspond to pCMT1 sequence.
[2]Truncated orf, 5'-end missing (it contains a number of frameshifts). Non functional.
[3]Partial sequence. The similar sequence probably extends beyond the indicated 5'-end.
[4]Truncated orf, 5'-end missing. Non functional. BlastP search reveals significant similarity to C-t of alpha protein of pUB110 (58% identical residues).

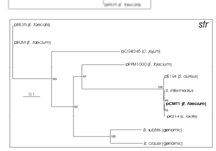
Genetic structure of the pCMT1 fragment containing *tetM*



pCMT1 (11958 bps)

Phylogenetic analysis of genes cat and str





References

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