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Characterization of Plasmid pML21 of *Enterococcus faecalis* ML21 from Koumiss

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Introduction

Plasmid mobilization has been recognized as the major mechanism for horizontal gene transfer in the evolution of antibiotic-resistant *Enterococcus faecalis* strains capable of causing human infection [1]. An investigation of plasmid distribution in *E. faecalis* and an analysis of their role in the dissemination of antimicrobial resistance can contribute to revealing the differences in the properties of clinically relevant versus probiotic strains of *E. faecalis*. Although a large number of *E. faecalis* plasmids have been sequenced and characterized, very few are from probiotic strains [2].

Many native plasmids from other lactic acid bacteria, such as lactobacilli and lactococci, have been used for the design of molecular tools, especially food-grade cloning and expression vectors [3, 4]. Although many *E. faecalis* strains isolated from food harbor abundant natural plasmids [5], only a few have been developed into valuable tools for biotechnological applications.

Enterococcus faecalis strain ML21 (CGMCC No. 6166) was originally isolated from the koumiss produced by nomadic families of the Ili pastoral area of Xinjiang Uyghur Autonomous Region, China. Susceptibility testing by the agar disk diffusion method showed that the isolate is susceptible to vancomycin and some other antibiotics (e.g., erythromycin, chloramphenicol, and ampicillin). This susceptibility is remarkably different from the vast majority of enterococci from clinical cases [6]. *E. faecalis* ML21 harbors a native plasmid, designated pML21. DNA sequencing on an ABI 3730XL DNA Analyzer (Applied Biosystems Inc., Foster City, CA) showed that this plasmid is 5,598 bp in size with 32.9 % G+C, and is predicted to carry five putative open reading frames (ORFs) (Fig. 1a). Sequence analysis of the replicon region (ORF1, ORF2, and the sequence upstream of ORF1) of pML21 revealed that it is a member of the pCI305 family of plasmids, belonging to the group of lactococcal theta-type replicons [8], which differ from the rep9 family replicon plasmids that are prevalent in clinical strains of *E. faecalis* [2]. We therefore speculated that horizontal gene transfer might have occurred between *E. faecalis* ML21 and lactococcal strains in this niche, as the fermented dairy product koumiss contains a complex microbial ecological community. The plasmid also contains an ε antitoxin component of the ε/ζ-type toxin–antitoxin system (encoded by ORF5) but interestingly, no cognate toxin gene was detected on this plasmid. Based on homology blast, pML21 ORF3 was predicted to encode an abortive infection phage resistance (AIPR) protein which is often found in restriction–modification system operons. ORF3 and ORF4 were found likely to be under the control of a specific bidirectional promoter in pML21. ORF4 was predicted to encode a hypothetical MerR family transcriptional regulator protein, but its function remains unknown. The relative copy number of this plasmid was estimated to be about 137.81 ± 3.20 copies per cell, as determined by quantitative real-time PCR [9], higher than that of other closely related plasmids [10, 11].

To develop molecular tools using pML21, an *Escherichia coli* lactic acid bacteria shuttle vector designated pML23e was constructed (Fig. 1b). This vector was able to transform all tested lactic acid bacteria strains, including *E. faecalis* ML21, *Lactococcus lactis* subsp. *cremoris* MG1363,

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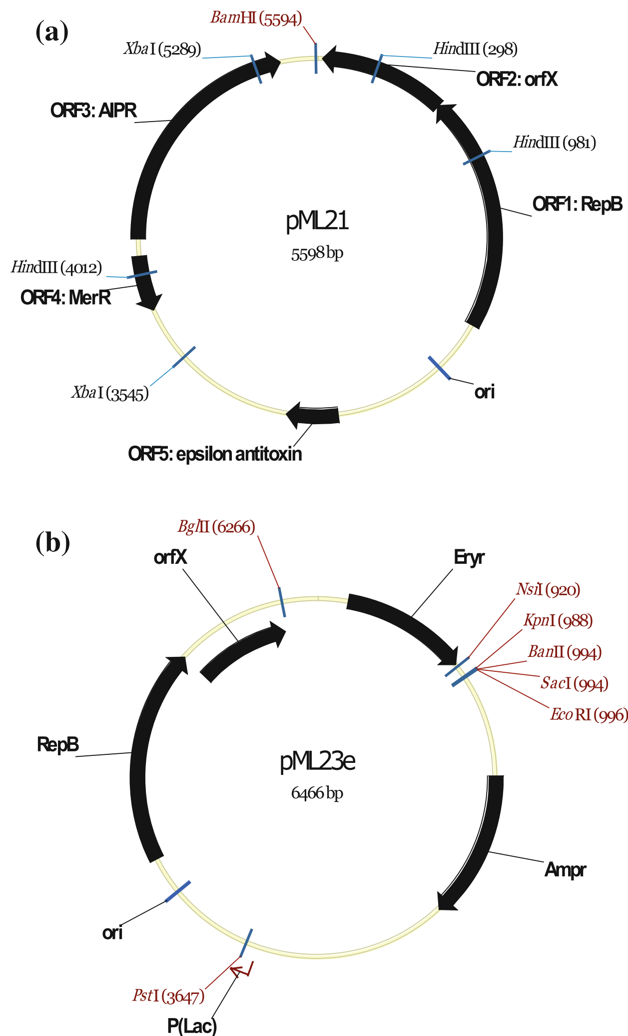

Fig. 1 Physical maps of pML21 (**a**) and pML23e (**b**). ORFs are shown as *arrows*. The unique restriction site *Bam*HI was used to linearize pML21 for construction of the sequencing vector; pML23e was constructed by linking a 2,631-bp predicted pML21 replicon containing a fragment generated by PCR using primers pML-F (5′-CCTGCAGAATCATTTATTGAGTCTCATTTC-3′, *Pst*I *underlined*) and pML-R (5′-TAGATCTTTTAATCAAAAGTAAACGTGCGG-3′, *Bgl*II *underlined*), and a 3,852-bp fragment containing the *E. coli* replicon and Ery^r and Amp^r elements from *E. coli*-*Bifidobacterium* shuttle vector pAM1 by PCR using primers pAM1-F (5′-CAGATCTGTCATGGCGTGGTAGATG-3′, *Bgl*II *underlined*) and pAM1-R (5′-ACCTGCAGGCATGCAAGCTTG-3′, *Pst*I *underlined*) [7]. The restriction sites used for cloning—*Nsi*I, *Kpn*I, *Sac*I, *Ban*II, and *Eco*RI—are shown

L. lactis subsp. *lactis* LM0230, *Lactobacillus acidophilus* 05-172, *Lactobacillus rhamnosus* NL24, *Lactobacillus paracasei* L14 and *Lactobacillus plantarum* PC23, by electroporation as described previously [12], with some modifications (i.e. using 30 % (m/v) PEG1500 to suspend competent cells and pulse with 12.5 kV/cm). Transformation efficiencies ranged from 3.1×10^1 to 3.5×10^4 transformants per microgram plasmid DNA, supporting the use of pML23e to perform genetic manipulations in lactic acid bacteria (Table 1). Furthermore, pML23e was stably maintained in *L. lactis* subsp. *cremoris* MG1363*, L. acidophilus* 05-172 and *L. plantarum* PC23 for 100 generations without selective pressure (Table 1). Taken together, our results indicate that the newly isolated high copy number plasmid pML21 has potential for use in plasmid construction for multi-host cloning or expression shuttle vectors; such vectors might be useful for food biotechnology applications of lactic acid bacteria.

Table 1 Host range and segregation stability of vector pML23e harboring the pML21 replicon

Strains	Reference or source	Transformation efficiency (cfu/μg plasmid DNA)	Antibiotic-resistant colonies (%)
E. faecalis ML21	This work	1.9×10^3	8 %
L. lactis subsp. *cremoris* MG1363	[13]	5.3×10^3	55.7 %
L. lactis subsp. *lactis* LM0230	[14]	9.4×10^1	ND
L. acidophilus 05-172	[15]	7.3×10^1	75.5 %
L. rhamnosus NL24	This work	3.1×10^1	ND
L. paracasei L14	CGMCC	3.5×10^4	30 %
L. plantarum PC23	This work	7.3×10^3	53.8 %

ND not detected

Nucleotide sequence accession number. The full nucleotide sequence of pML21 has been submitted to the GenBank database and assigned accession no. JX023543.

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