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Differences in the expression of cell envelope proteinases (CEP) in two *Lactobacillus paracasei* probiotic strains

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One sentence summary: This work dissects the molecular regulation of the cell wall proteinase and related genes in two probiotic strains of *Lactobacillus paracasei*.

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ABSTRACT

Proteinase PrtP (EC:3.4.21.96) is a cell envelope proteinase (CEP) highly expressed in the probiotic strain *Lactobacillus paracasei* BL312(VSL#3) that accounts for its anti-inflammatory properties. The main aim of this work is to understand differences in CEP expression between this strain and *L. paracasei* BL23. Hence, differences in the regulation by amino acid sources of four proteinase related genes (*prtP*, *prsA*, *prtR1* and *prtR2*) were determined by RT-qPCR in BL312(VSL#3) and BL23 using as a reference BL368, a BL23 derepressed mutant lacking the response regulator (RR) PrcR. BL312(VSL#3) showed greater expression of *prtP* (2- to 3-fold) than BL23, and *prtP* was highly repressed by peptone in both strains. Two other putative CEP genes, *prtR1* and *prtR2*, showed a low expression profile. Interestingly, when the *prsA-prtP* promoter region from both strains, and deleted mutants, were cloned in vector pT1GR, expression of the *gfp* and *mrfp* fluorescent reporters was always repressed in BL23 (high or low peptone) and derepressed in BL368, revealing an interesting mechanism of regulation affecting specifically to this promoter. In conclusion, BL312(VSL#3) has higher expression of *prtP* and other CEP related genes than BL23, that could respond to a natural deregulation in this strain, possibly independent from the RR PrcR.

Keywords: Proteinases; probiotic; *Lactobacillus paracasei*; regulation features; expression vector; RT-qPCR

INTRODUCTION

Cell envelope proteinases (CEP) from lactic acid bacteria (LAB), generically called lactocepins, constitute a family of unusually large proteins that belong to the group of cell wall-anchored subtilisin-like PII-type proteinases (EC:3.4.21.96). In *Lactococcus lactis* and closely related species, CEP was designated PrtP and it was studied for their relevance in cheese production, as it plays an important role in the physicochemical and sensorial

evolution of cheese and other dairy products (Law and Haandrikman 1997; Broadbent *et al.* 1998, 2002; Chen *et al.* 2017). In the last decade, these proteinases have been highlighted by their potential functional activity, for instance generating peptides that inhibit the angiotensin-converting enzyme (ACE, reviewed by Raveschot *et al.* (2018)). They have also been related to the potential reduction of milk allergies by destroying allergens of β -lactoglobulin (Pescuma *et al.* 2015) and α S1-caseins

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(El-Ghaish *et al.* 2011). Furthermore, lactocepin from the *Lactobacillus paracasei* strain found in the commercial probiotic mixture VSL#3 selectively degraded IP-10, resulting in a significantly reduced inflammatory profile and lymphocyte recruitment after intraperitoneal injection in a murine ileitis model (von Schillde *et al.* 2012). In fact, these activities could be the mechanisms underlying the observed anti-allergic and anti-inflammatory effects of dairy products containing lactobacilli (Lee *et al.* 2007; Gobbato, Rachid and Perdigon 2008; Chaves, Perdigon and de LeBlanc 2011; Sheikhi *et al.* 2012). For this reason, knowledge on factors that regulate this enzymatic activity is essential for the selection of proteinase proficient strains and the improvement of probiotic lactobacilli performance, particularly in the *L. casei/paracasei* taxon.

In *L. paracasei* BL23, the genes encoding PrtP (*prtP*, LCABL.24 520) and its maturase PrtM (*prsA*, LCABL.24 530) are adjacent and transcribed in opposite direction, hence the promoters for both genes are in the *prsA-prtP* intergenic region. When this promoter region was cloned upstream the β -glucuronosidase gene (*gusA*) reporter gene a repressing effect was found on a peptide rich medium (containing casein) on the gene expression of *prtP* and *prsA* (Pastar *et al.* 2007). A more recent study described that the promoter region of *prtP* and *prsA* is recognized by the response regulator (RR) PrcR. PrcR acts as a repressor of the promoter activity on peptide rich growth media and, interestingly, it binds to the promoter region of those genes even in the absence of the canonical phosphate supplier, acetyl-P (Alcantara *et al.* 2016).

Furthermore, five subgroups of CEPs have been described in LAB according to their domain composition: PrtB from *Lactobacillus delbrueckii* subsp. *bulgaricus*, PrtH from *Lactobacillus helveticus*, PrtP from *L. lactis* and *Lactobacillus paracasei*, PrtR from *Lactobacillus rhamnosus* and PrtS from *Streptococcus thermophilus* (Siezen 1999; Fernandez-Espla *et al.* 2000; Pastar *et al.* 2003; Savijoki, Ingmer and Varmanen 2006; Alcantara *et al.* 2016). After detailed analysis of *L. paracasei* BL23 genome a cluster of two proteinase genes was found that encoded proteins homologous to PrtR from *Lactobacillus rhamnosus* (Pastar *et al.* 2003; Muñoz-Provencio *et al.* 2012), *prtR1* (LCALB-0 5520) and *prtR2* (LCALB 0 5530). However, little is known about their function or expression in *L. paracasei*.

This work explored the regulatory effect of amino acids on *prtP* and *prsA* genes in the probiotic and laboratory strain *L. paracasei* BL23, using a highly proteolytic deregulated mutant (BL368) and a commercial probiotic isolate (*L. paracasei* VSL#3). For this purpose, we used RT-qPCR and designed a new expression vector carrying the genes encoding fluorescent reporters GFPuv and RFP.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Strain *L. paracasei* BL23 is a laboratory strain (Acedo-Felix and Perez-Martinez 2003) and *L. paracasei* BL368 is a derivative mutant of BL23 in the RR PrcR that displayed faster milk clotting and acidification properties (Alcantara *et al.* 2016). *L. paracasei* from the probiotic mixture VSL#3 (here BL312) was a generous gift from Dr. Claudio De Simone (L'Aquila, Italy). All lactobacilli were grown under static conditions in MRS medium (Difco) at 37°C. *L. paracasei* strains carrying expression vectors were grown on MRS broth or agar with 5 μ g/mL erythromycin. For the assays with high (20 g/L) and low (1 g/L) peptone as main source of peptides and amino acids, strains were grown on a semi-defined medium (SDM) modified from Morishita *et al.* (1981), from which

all sources of amino acids were eliminated except yeast extract. The rest of the components were identical to the original recipe containing per litre: 5 g yeast extract (Difco Laboratories, Detroit, MI, USA), 5 g D-glucose, 1.7 g sodium acetate, 2 g ammonium citrate, 3 g KH₂PO₄, 3 g K₂HPO₄, 0.575 g MgSO₄·7H₂O, 0.034 g FeSO₄·7H₂O, 0.12 g MnSO₄·2H₂O, 0.5 g L-cysteine hydrochloride and 1 g Tween 80; final pH was adjusted to 7.2. *E. coli* DH10B and DH5 α were the intermediate cloning hosts, and they were grown under aerobic conditions at 37°C in LB Broth (Difco) or LB with 2% agar containing 300 μ g/mL erythromycin. In case of pT1Nx derivatives, *Lactococcus lactis* subsp. *cremoris* MG1363 was used as intermediate host to transform vector ligations. Plasmid carrying strains were grown on M17 (Difco) with 5 g/l glucose (GM17) liquid or with 1.8% agar and 5 μ g/mL erythromycin.

DNA manipulation and cloning

The close phylogenetic relationship between strain BL312(VSL#3) and BL23 allowed designing oligonucleotides to sequence *prtP* and *prsA* genes in order to obtain the complete sequence of the *prtP-prsA* genes and intergenic region by chromosome walking. The primers used and the complete sequence are described in Table S1 (Supporting Information).

The reporter genes *gfp* and *mrfp* from vector pTLGR (García-Cayuela *et al.* 2012), that express respectively green fluorescent and red fluorescent proteins, were subcloned. Specific details on restriction enzymes used, blunt end generation with Klenow enzyme or cloning by PCR amplification are described in Fig. 1. Briefly, first the expression cassette *gfp-mrfp* was subcloned in the *Bam*HI/*Sma*I sites of vector pIAV7 (Perez-Arellano *et al.* 2003) made blunt with Klenow enzyme yielding pIA-GR, which would facilitate transformation in *E. coli* and a stable maintenance in lactobacilli. All promoter versions – including deleted mutants – were PCR amplified and ligated to *Bam*HI-digested pIA-GR, and the whole cassette – promoter and reporter genes – were then subcloned in the *Eco*RI-Klenow blunt/*Spe*I sites of the expression vector pT1NX (Steidler *et al.* 2000). Plasmid pT1GR is identical to pT1GR::PprsA-PprtP but with no promoter sequences between the reporter genes, and it was obtained by direct amplification of the *gfp-mrfp* cassette from pIA-GR, that was then cloned in pT1NX (*Eco*RI-Klenow blunt/*Spe*I).

Expression analysis of fluorescent reporters

The expression of the reporter genes was determined in 96-well plates during 12 h incubation in the corresponding medium inoculated with the different strains at least in triplicate using a POLARstar plate reader instrument (BMG Labtech, Offenburg, Germany) equipped with a temperature-controlled plate and shaker. GFP fluorescence was determined with excitation/emission filters of 485/520 nm and the Cherry mRFP fluorescence was measured using excitation/emission filters of 540/612 nm. Automatic recording of data was set up every 5 min and fluorescence was determined for all strains and conditions during 12 h (720 min); if maximum was reached before 12 h this was the value selected for graphical representation of relative fluorescence. Differences were calculated relative to the control vector (pT1-TGR) bearing both fluorescence genes but lacking any promoter sequence. Fluorescence values were corrected for growth (absorbance at 590 nm) at the corresponding time points. A general linear model was applied for the analysis of variance (ANOVA) individually of green and red fluorescence data in the two strains BL23 and BL368, using IBM SPSS package software. Significance level was calculated according to HSD Tukey test.

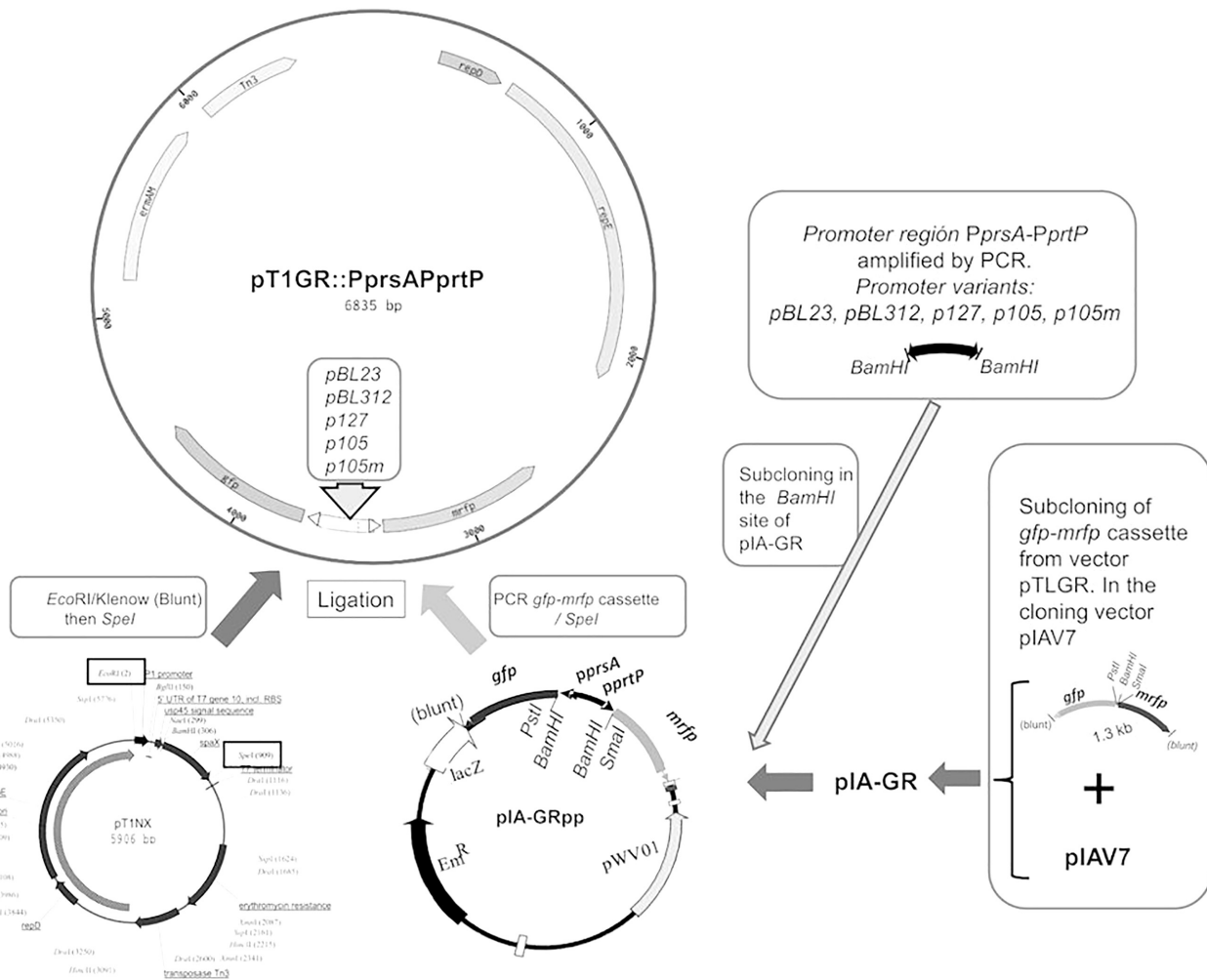


Figure 1. Summarized diagram of the steps followed for the construction of the reporter plasmids used in this work. For a more detailed description check Figure S1 (Supporting Information).

RNA isolation and RT-qPCR

RNA extraction and the general procedure for RT-qPCR was as described before (Landete et al. 2010). Briefly, RNA isolation was performed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA integrity was confirmed using the Experion automated electrophoresis system (Bio-Rad, Hercules, CA, USA). Contaminating genomic DNA was digested using DNaseI (Ref. AMP-D1, Sigma, St. Louis, MO, USA) and cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer. A total of four housekeeping genes, *ileS*, *lepA*, *pcrA* and *pyrG*, were tested to normalize gene expression, that were respectively encoding isoleucyl-tRNA synthetase, GTP-binding protein, ATP-dependent DNA helicase and CTP synthetase. The primer sequences for PrtR1 and PrtR2 were designed using the Primer Blast tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and are listed in Table 2. Gene expression ratios were calculated using the software tool REST based on the efficiency-corrected method (Pfaffl, Horgan and Dempfle 2002). When bacterial strains were grown under different peptone concentrations, *lepA* and *pyrG* showed no stable gene expression for which they were disregarded in their respective calculations.

RESULTS

Transcription analysis of CEP related genes in *L. paracasei* strains

The repressing effect by rich amino acid sources on proteinase activity has already been studied in *L. paracasei* (Pastar et al. 2007; Alcantara et al. 2016); however, differences between probiotic strains at transcriptional level had not been described, as it is the case of *L. paracasei* BL23 and *L. paracasei* BL312(VSL#3). For this purpose, expression of the chromosomal genes encoding CEP related genes (*prtP*, *prsA*, *prtR1* and *prtR2*) was analysed by RT-qPCR. In addition to *prtP* and *prsA*, encoding PrtP and the associated maturase PrtM, two new CEP paralogous genes found in the genome of BL23 were included. They showed great similarity to *L. rhamnosus* *prtR*, *prtR1* (LCABL.0 5330) and *prtR2* (LCABL.0 5320) (Muñoz-Provencio et al. 2012) and similar counterparts were present in BL312 (VSL#3), as deduced by the amplification with specific qPCR primers. Differential expression of all CEP related genes (*prtP*, *prsA*, *prtR1* and *prtR2*) was tested on 1 and 20 g/L peptone (Table 1). Since SDM is a poor medium, 1 g/L peptone was required to support growth of BL23, and it did not activate PrcR dependent repression, as shown by the results. In strains BL312(VSL#3) and BL23 expression of

Table 1. Relative gene expression ratios of the of genes *prtP*, *prsA*, *prtR1* and *prtR2* in strains BL23, BL312(VSL#3) and BL368 grown with different peptone concentrations. The upper part of the table shows intra-strain comparisons of gene expression due to peptone concentration. The lower part shows gene expression between strains relative to strain BL23.

Peptone	Strain	<i>prtP</i>		<i>prsA</i>		<i>prtR1</i>		<i>prtR2</i>	
		Exp. rate	Std. Err range	Exp. rate	Std. Error range	Exp. rate	Std. Error range	Exp. rate	Std. Error range
1 g/L	BL23	1		1		1		1	
20 g/L		0.178*	0.173–0.183	0.937	0.872–1.006	0.721	0.688–0.756	0.888	0.777–1.019
1 g/L	BL312	1		1		1		1	
20 g/L		0.313*	0.299–0.328	0.738*	0.709–0.768	0.688*	0.666–0.712	0.854*	0.795–0.917
1 g/L	BL368	1		1		1		1	
20 g/L		1.054	1.026–1.082	2.058	1.992–2.128	0.809*	0.790–0.829	1.021*	1.013–1.029
1 g/L	BL23	1		1		1		1	
	BL312	2.008	1.915–2.107	2.674	2.486–2.880	6.695*	6.380–7.034	57.467*	50.269–66.023
	BL368	9.434*	9.180–9.697	1.093	1.017–1.175	0.966	0.921–1.013	1.539	1.347–1.759
20 g/L	BL23	1		1		1		1	
	BL312	3.523	3.470–3.577	2.106*	2.092–2.120	6.390*	6.366–6.413	55.217*	52.500–58.082
	BL368	55.767*	54.271–57.317	2.401	2.325–2.480	1.084	1.059–1.109	1.769*	1.682–1.861

(*) P value \leq 0.05.

Table 2. Primers used in this study.

Primer name	Sequence 5'–3'	Features	Ref.
Pro1	GGCGAATTCCAAGCAAAGCGGC	EcoRI	This work
Pro2	CATCGAGCTCGCCTTAGCATCAG	SacI	This work
Prom.prt.F	AATTGGATCCTGAAATGCTTACAGTAAACG	BamHI	This work
Prom.prt.R	AATTGGATCCATAACCCGGGTTCCCAAAAGTTTCAGAACC	BamHI- SmaI	This work
PprtP_cor	CGGGATCCAAGATTAATAATTTTCGTTGAATCTGT	BamHI	This work
PprtP_corm	CGGGATCCTTGAATGTAATATACGTTGAATCTG	BamHI,	This work
M94F	CGGAATTCGCGGCTCTCCTCTGAAATGCTT	BamHI	Pastar et al. (2007)
M94R	CGGGATCCCGCATTATTAGAAAATGAGTATTTGC	BamHI	Pastar et al. (2007)
P127F	CGGGATCCCGCAGATAGAAAATTAATAAAAAGATTAA	BamHI	Pastar et al. (2007)
P127R	CGGAATTCGCGCAATACCCTCCACTTCCC	EcoRI	Pastar et al. (2007)
TLGR.compl.F	TAGTCGACTAGTCTCGAGCCTATTTGTATAGTTCATCC	Sall-SpeI-XhoI	This work
TLGR.compl.R	GCTTCTCAAGGGCATCGGTTCG		This work
PrtP for	CTTCTGAACGCTGGCATC	RT-qPCR	Alcantara et al. (2016)
PrtP rev	GCCATTATCTGATGAACAGG	RT-qPCR	Alcantara et al. (2016)
PrtM for	GCCCAAAGTGACTGTCCAGC	RT-qPCR	Alcantara et al. (2016)
PrtM rev	TTCGCAAGCGTGGCAAAATC	RT-qPCR	Alcantara et al. (2016)
PrtR1 for	AAACACCAGCGTCACGAGTC	RT-qPCR	This work
PrtR1 rev	GCGGTTGCACATCCCTTGT	RT-qPCR	This work
PrtR2 for	ATGGTGCCTGCAAAGGATGG	RT-qPCR	This work
PrtR2 rev	GGCCACTGGTAAGCTCATGG	RT-qPCR	This work

prtP was remarkably repressed and all other genes were moderately affected by amino acids. As expected, peptone did not affect expression of *prtP* in the deregulated mutant BL368. Under conditions of low amino acid supply, the greatest expression ratios corresponded to *prtP* in BL368 and *prtR1* and *prtR2* in BL312(VSL#3). At the highest peptone concentration, *prtP* gene expression in strain BL312(VSL#3) decreased but remained proportionally higher than in BL23, thus indicating that the higher activity/expression of *prtP* in BL312(VSL#3) may be related to other strain specific factors, not to a deregulation by nitrogen sources. On the other hand, BL368 showed a much greater expression of *prtP* but not of the other genes, indicating that they must be moderately regulated by PrcR, as previously reported (Alcantara et al. 2016).

The relatively higher expression of *prtR1* and *prtR2* in BL312(VSL#3) could be interpreted as if these genes were highly expressed; however, the careful observation of RT-qPCR data showed that *prtR1* and *prtR2* normally appeared at high Cts. Since *prsA* showed relatively uniform expression, it was considered a suitable internal reference to compare differences in the expression of all the genes within each strain (Table S2, Supporting Information) the greatest differences were detected on 1 g/L peptone, where *prtP* expression was 7- to 10-fold greater than *prsA* in strains BL23 and BL312 and, in BL368, *prtP* reached 83.8-fold the expression of *prsA*, suggesting that *prtP* suffered a much tighter control by PrcR, than *prsA*. Also, these numbers showed a poor relative expression of *prtR1* and *prtR2*. Only in strain BL312 grown on high amino acid concentration, expres-

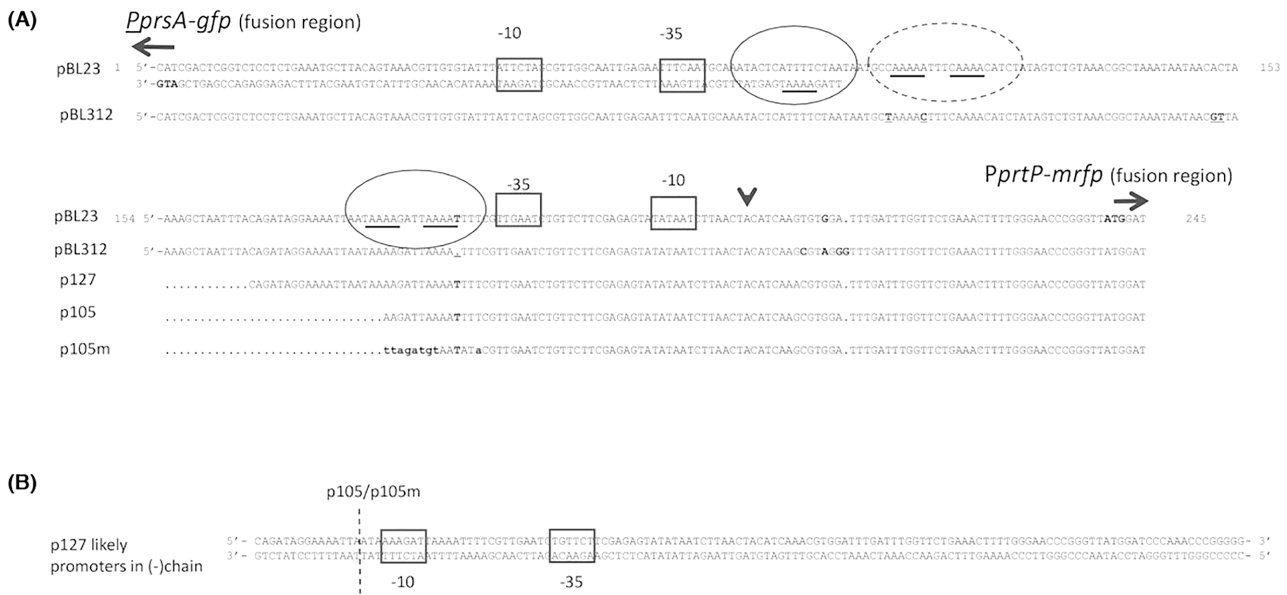


Figure 2. Nucleotide sequence of the *prsA-prtP* intergenic region cloned in the expression vector pT1GR, that contains the promoter regions (-10 and -35) framed in square boxes. Panel A shows the sequences of the promoters tested in the reporter vector with sequence differences highlighted in bold, where the fusion regions with the reporter genes are indicated by horizontal arrows, the transcriptional start point is shown by a vertical arrow and the possible PrcR dependent regulatory regions are underlined and surrounded by ellipses. In pBL23, an uncharacterized regulatory region of *prtP* is shown in the (-)chain surrounded by a dotted ellipse. Panel B shows the putative promoter region found in the (-)chain that would explain the expression of *gfp* in the deleted promoter versions.

sion of *prtR2* was comparable to *prtP* (*prtR2* 2.2 and *prtP* 3.2-fold, relative to *prsA*).

Cloning and mutation of the *prsA-prtP* intergenic region containing the divergent promoters

Since *prtP* is the gene with highest expression in these probiotic strains, the promoter structure of the *prsA-prtP* intergenic region from BL23 and BL312(VSL#3) was PCR amplified and cloned in specially designed vectors with divergent reporter genes. Reporter genes were obtained from vector pTLGR (García-Cayuela et al. 2012), so that the promoters could be tested by the expression of the green fluorescent protein (GFP) from the gene *gfp^{UV}* and the red fluorescent protein (RFP) from *mrfp*. In order to improve the stability and replicative potential of the vectors in lactobacilli, the complete expression cassette was first cloned in the wide host range plasmid pIAV7, and later subcloned in vector pT1NX (Steidler et al. 2000) to optimize expression and stability (Fig. 1 and Figure S1, Supporting Information). Vector pT1GR was the new reporter without promoter, and pT1GR::PprsAPprtP was the general name for the reporter plasmid carrying the intergenic region *prsA-prtP* of approx. 260 nt from BL23, here called pBL23, or BL312(VSL#3) (pBL312) (Fig. 2). In addition, three versions of the pBL23 promoter were generated by deletion in the 5' region lacking the putative -10 and -35 regions of *prsA* and the PrcR regulatory sequences (AAAA), but that included the promoter region and transcriptional start of the gene *prtP* as previously characterised (p127) (Pastar et al. 2007) (Fig. 2). The two other constructs -promoters p105 and p105m- lacked possible regulatory region overlapping the *prtP* promoter (Alcantara et al. 2016). All five versions of the *prsA-prtP* promoter (pBL23, pBL312, p127, p105 and p105m) with their known regulatory regions, promoter sites and sequence differences are detailed in Fig. 2.

Expression analysis from the fluorescent reporter genes

All five promoter versions described above were cloned in the reporter vector pT1GR, in which the expression of the gene encoding RFP (*mrfp*) would be under the control of the *prtP* promoter (PprtP) and GFP gene (*gfp*) was controlled by the *prsA* promoter (PprsA). Red and green fluorescence were monitored when plasmids were transformed in BL23 and in the deregulated mutant BL368 lacking the RR PrcR. Expression of both reporters was also determined in all recombinant strains with low and high concentrations of amino acids (1 g/L and 20 g/L peptone) (Fig. 3A and B). In the wild type strain BL23, expression of RFP from the complete fragments, pBL23 and pBL312 was similar (no statistical difference), and the highest expression was found in promoters p127 and p105, thus indicating at least a partial release of repression in the deleted mutants. Nevertheless, differences between cells growing in medium with low or high peptone content were not significant (Fig. 3A). When reporter plasmids were introduced in the deregulated strain BL368, expression of RFP from all promoter versions was much higher than in wild type BL23 and followed a different pattern; here pBL312 induced a significantly lower expression than pBL23 and similar to deleted p105 (Fig. 3A). In this case, expression from pBL23 almost reached p127.

In relation to the expression of GFP from the promoter of *prsA* (PprsA), ANOVA showed that there was no significant effect of peptone on BL23, but expression decreased significantly in BL368. Little differences could be detected between pBL23, pBL312 and p127 in BL23, but there was a pronounced strain effect as GFP expression from the promoters pBL23 and pBL312 was remarkably derepressed in BL368 (Fig. 3B). This suggested a direct regulation of PrcR on PprsA, possibly through a partial consensus AAAA identified close to the putative -35 promoter region (Fig. 2B). Surprisingly, all the deleted promoters allowed

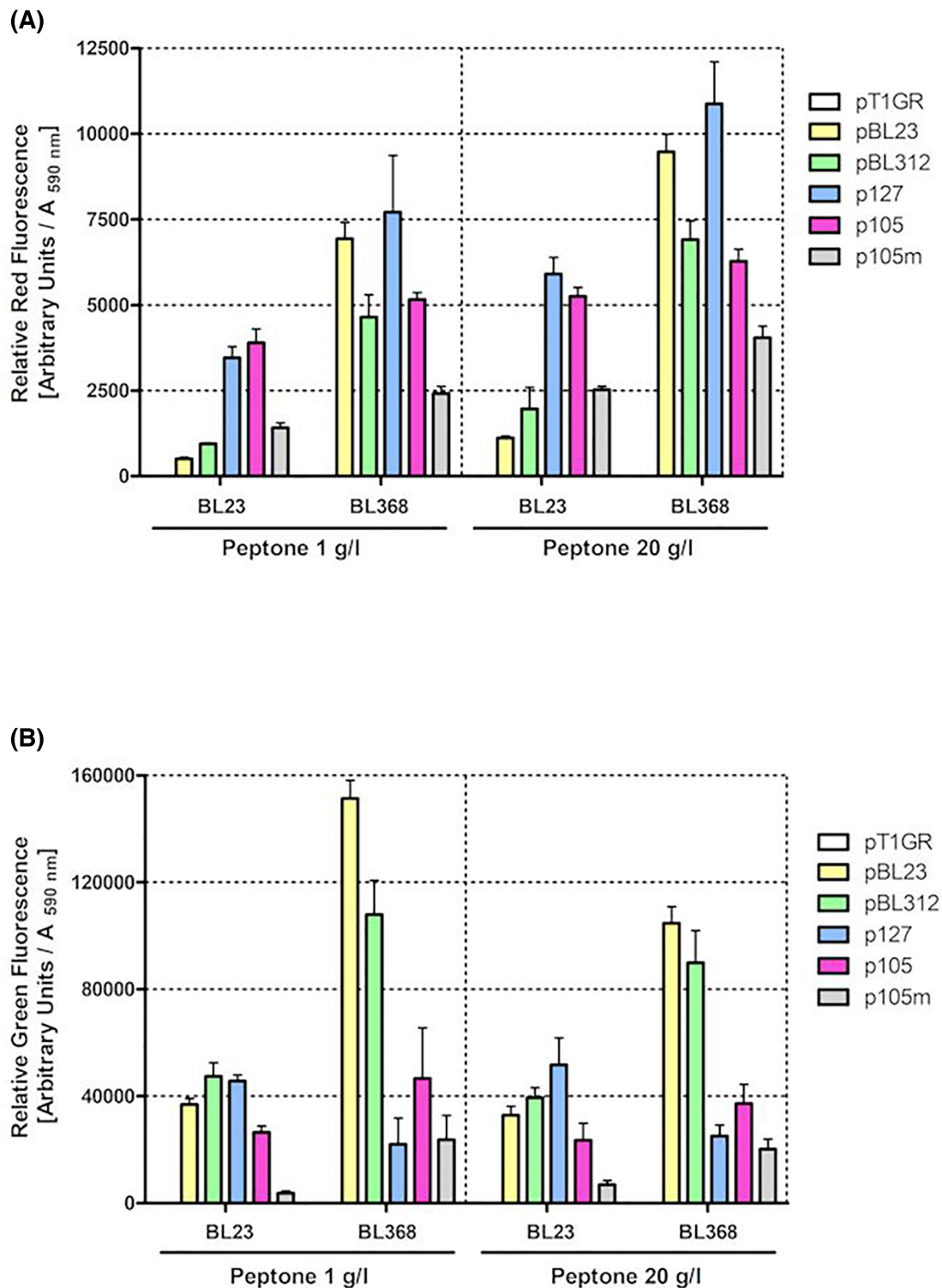


Figure 3. Relative fluorescence generated by the expression of the gene encoding the red fluorescent protein, mRFP, from the *PprtP* promoter (A) and the green fluorescent protein, GFP, from *PprsA* promoter in the reporter plasmid pT1GR. Strains *L. paracasei* BL23 and BL368 were transformed with pT1GR carrying different forms of the *prsA-prtP* promoter region: pBL23, pBL312, p127, p105 and p105m. For the sake of clarity, only the names of the promoters appear in the abscissa axes. Host strains (BL23 or BL368) are indicated in the histograms over the group of columns corresponding to the different promoters cloned in pT1GR. All strains were grown on SDM supplemented with 1 and 20 g/l peptone. ANOVA showed that for RFP expression (Panel A) in BL23 there was no significant difference between samples, with exception of pBL23-pBL312 and p127-p105, and pBL312-p105 in BL368 ($P > 0.01$). In case of GFP (Panel B) expression in BL23 revealed some significant difference between pT1GR-p105m and pBL312-p127 (p between 0.01–0.05) and in BL368 only p127-p105m were not significantly different ($P > 0.01$).

transcription of GFP, possibly because of the putative consensus -10 and -35 region identified. Its possible functionality was confirmed by the poorer expression found in *p105m* (Fig. 2B). All three deleted promoters did not show differences in expression due to the host strain or the presence of peptone.

DISCUSSION

Probiotic activity relies in a variety of mechanisms of action that are steadily being disclosed. Some of the probiotic mechanisms require the direct interaction of bacteria or bacterial components with the host cells like secreted surface muramidases, cell wall peptidoglycan, surface layer proteins or pili (Fernandez et al. 2011; Sanders et al. 2018), among others. Proteolytic activity of lactobacilli has been shown to specifically hydrolyse allergenic peptides of β -lactoglobulin (Pescuma et al. 2015) and α S1-caseins (El-Ghaish et al. 2011). Proteinases (CEP) from certain *Lactobacillus* strains are able to release peptides from caseins inhibiting the angiotensin-converting enzyme (ACE), hence regulating hypertension (Fuglsang et al. 2003). In addition, CEP from *L. paracasei* VSL#3 cleaves specifically the proinflammatory chemokine IP10 decreasing inflammatory signals in the mucosa of mice, possibly as a niche survival strategy (von Schillde et al. 2012). In general, CEP in lactic acid bacteria are repressed in the presence of sources of amino acids and short peptides (Hebert, Raya and De Giori 2000; Miladinov, Kuipers and Topisirovi 2001; Pastar et al. 2007; Alcantara et al. 2016). In this work features of the *prsA-prtP* promoter region of two *L. paracasei* probiotic strains have been compared using red and green fluorescence reporter proteins and quantified expression of *prtP*, *prsA*, *prtR1* and *prtR2* by RT-qPCR. Despite that such proteins may be sensitive to acidic pH, strains and growth conditions were identical, hence results may be useful for comparative purposes. Strains used were the *L. paracasei* isolate from the fully characterized commercial probiotic mixture VSL#3 (here BL312) (Douillard et al. 2018) and *L. paracasei* BL23, a strain with probiotic activity, in which two proteins have been characterized with likely antiapoptotic and epithelium repairing properties, CmuA (P40) and CmuB (P75) (Bäuerl et al. 2010). Differences in expression of *prtP* gene between different strains coincided with previously reported differences in proteinase activities between BL23 and BL312(VSL#3) (von Schillde et al. 2012) and between BL23 and BL368 (Alcantara et al. 2016). Strains BL368 and BL312(VSL#3) showed a moderate derepression of *prsA*, encoding the maturase PrtM. The presence of 20 g/L peptone had a prominent effect on the expression of some of the CEP related genes in BL312 and *prtP* in BL23.

In contrast, cloning of the promoters in plasmid pT1GR did not show evident repression of the reporter RFP expressed from the *prtP* promoter, as shown by RT-qPCR. PrcR regulates a number of genes in *L. paracasei* in response to amino acid and peptide sources, but *prtP* promoter had some particular features (Alcantara et al. 2016) that could help understanding the results of the fluorescent expression vector. In this previous work, Alcantara et al. (2016) showed that this promoter was repressed by peptide sources, but the dephosphorylated form of PrcR could also bind to it and, as consequence, it might bind the *prtP* promoter in the absence of amino acid sources. This suggested that this promoter region could have a more complex regulation than other promoters regulated by the RR PrcR, possibly affected by the presence of multiple copies of the promoter when present in a plasmid. It is known that multiple copies of a regulated DNA region can titrate interacting elements (Brewster et al. 2014). Here, it was evident that PrcR still binds the promoters born in the plasmid (much higher expression in BL368 than in BL23), but

the lack of repression by peptone indicates that there could still be another element that regulate PrcR phosphorylation –or an unknown PrcR effector-, that is titrated out by large numbers of the promoter fragment. This suggested that this promoter region could have a more complex regulation than other promoters regulated by the RR PrcR. Considering these special regulatory features, the monitoring of RFP expression suggested that the deleted promoter *p127* was partially derepressed because it excluded one of the regulatory elements AAAA recognized by the repressor PrcR in the *prsA-prtP* promoter region (Fig. 2), yielding a higher expression than the complete fragment (*pBL23*) in the wild type strain BL23. In BL368 –lacking the repressor-, RFP reached its highest level, and *pBL23* was fully derepressed, yielding almost as much red fluorescence as *p127*. The defective 5' region of *p105* and *p105m* rendered lack of regulation but poorer expression than *p127*.

An important aim of this work was to investigate differences between the *prsA-prtP* promoters of the probiotic strains BL23 and BL312(VSL#3). They differed in just 7 nucleotide differences and two single base insertion/deletions (Fig. 2) that did not generate significant differences of RFP expression from *pBL23* and *pBL312* in the wild type strain BL23. In the PrcR mutant (BL368), *pBL312* was also derepressed indicating the same type of regulation, but total expression was lower than from *pBL23*, suggesting that the mutations may indeed affect the efficacy of expression in BL368 that has BL23 genetic background. This may indicate that the higher CEP activity (von Schillde et al. 2012) and mRNA synthesis of *prtP* gene in BL312 must be due to naturally higher expression levels not only dependent of a PrcR deregulation in this strain, this higher transcription rate was also affecting the other CEP related genes, *prsA*, *prtR1* and *prtR2*.

Regulation of the *PprsA* promoter has also been monitored through the expression of GFP and, again, a similar lack of repression by peptone was found, possibly by the same factors that affected *PprtP*. In BL23, expression of *gfp* from *pBL312* was significantly greater than from its native strain promoter *pBL23*, also RT-qPCR data showed that BL312(VSL#3) had higher expression of *prsA*. In BL368 both native promoters, *pBL23* and *pBL312*, showed a pronounced derepression of GFP expression. The deleted promoters did not contribute much in this part of the study, but for the fact that a new unforeseen promoter -10 and -35 in *p127* provided high constitutive expression under both culture conditions and in both strains.

The activity and the role of PrtR paralogs in *L. paracasei* will require further investigation, but the relative expression of all the genes -within each strain- revealed a generalized low expression profile of *prtR1* and *prtR2* compared to *prtP*. The lack of repression of *prtR2* rendered apparent high ratio relative to *prtP* in BL312 on high peptone concentration. Future studies will determine if PrtR1 and PrtR2 are just remains of a common ancestor with *Lactobacillus rhamnosus*, or if they have a metabolic role.

In conclusion, this study highlighted some features of the *prtP-prsA* promoters and differences between two probiotic strains in CEP and genes related. The effect of peptone on PrtP expression has been reported in previous studies (Pastar et al. 2007; Alcantara et al. 2016); here promoters from two probiotics (BL23 and BL312(VSL#3)) showed a remarkable repression of *prtP*, but only a small effect on *prtR1* and *prtR2* mRNA synthesis. When increasing the peptone concentration, gene expression in strain BL312(VSL#3) decreased but remained proportionally higher than BL23, thus indicating that the higher activity/expression of *prtP* in BL312(VSL#3) may be related to other strain specific factors, not to the deregulation by

nitrogen sources. Cloning of the promoters and deleted mutants in an expression vector with divergent fluorescent markers confirmed that the regulation of PprtP-PprsA promoter by PrcR could be complex as suggested before, as PrcR would still bind the promoter under low amino acid supply and high concentration of target DNA.

Understanding the regulation of very relevant functional properties, such as CEP activity, will be essential to control the biological effects, to exploit the potential benefits of these bacteria and for the selection of probiotic strains.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSLE](https://femsle.onlinelibrary.wiley.com/doi/10.1111/femsle.13671) online.

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