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The expression of propionicin PLG-1 gene (plg-1) by lactic starters[1]

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Propionicin PLG-1 is a bacteriocin produced by *Propionibacterium thoenii* P127. Such bacteriocin inhibits wide range of food-borne pathogens such as pathogenic *Escherichia coli, Pseudomonas aeruginosa, Vibrio parahaemolyticus, Yersinia enterocolitica* and a strain of *Corynebacterium* sp. In the present study, *plg-1* gene expressing propionicin PLG-1 was isolated, sequenced for the first time and the resulting sequence was analysed using several web-based bioinformatics programs. The PCR product containing *plg-1* gene was transferred to different lactic acid bacterial (LAB) strains using pLEB590 as a cloning vector to give the modified vector pLEBPLG-1. LAB transformants showed an antimicrobial activity against *Esch. coli* DH5α (most affected strain), *Listeria monocytogenes* 18116, and *Salmonella enterica* 25566 as model pathogenic strains. Such LAB transformants can be used in dairy industry to control the food-borne pathogens that are largely distributed worldwide and to feed schoolchildren in the poor countries where dangerous epidemic diseases and diarrhoea prevail.

Keywords: Propionicin PLG-1, *plg*-1 gene, lactic acid bacteria (LAB), food-borne pathogens.

Propionibacterium thoenii P127 (ATCC 4874) was discovered as a bacteriocin producer strain during screening of several propionic acid bacteria isolates (Grinstead, 1989). Previously, it was not applicable to obtain bacteriocin production in liquid medium, but the bacteriocin was extracted from solid medium on which strain P127 was grown (Lyon & Glatz, 1991). Such extracted bacteriocin did not exhibit any activity against strains of *P. freudenreichii*, but was active against *P. thoenii, P. jensenii* and *P. acidipropionici*. The viable count of the sensitive strain *P. thoenii* P5 was reduced by 99·6% using this extract. The activity of the extract was protease-sensitive, and moderately thermostable (80 °C, 30 min). Furthermore, the extract was inhibitory for some Gram-negative bacteria, yeasts and moulds. Several typical food-borne pathogens and spoilage microorganisms belonging to the genera *Bacillus, Staphylococcus, Clostridium, Yersinia*, and two species of *Salmonella* were found to be insensitive (Lyon & Glatz, 1991).

The bacteriocin was purified by several methods (Lyon & Glatz, 1991, 1993; Paik & Glatz, 1995). Generally, the cells were grown for 14 d in sodium lactate broth at 32 °C and a constant pH 7·0. Purification of the bacteriocin involved ammonium sulphate precipitation of the culture supernatant, binding of precipitated proteins to a cation exchanger at pH 6·5 and elution by a salt gradient. The active fraction was thereafter concentrated by dialysis against solid polyethylene glycol and subjected to reverse phase HPLC. The activity was eluted late in the elution gradient, indicating a highly hydrophobic compound. The recovery of activity was close to 100% for the whole procedure, although minor components were detected. The active fractions from the reverse phase chromatography step were all dominated by a 9700 Da peptide. The peptide was estimated to contain 99 amino acids with a calculated molecular weight of 9328 Da and N-terminal sequencing gave the following sequence: N-V-D-A(T)-R-T(C)-A(T)-R-T(A)-P. This sequence did not share homology with any protein in the sequence databases. From the data presented by Paik & Glatz (1995), it could be expected that the culture produced about 2 mg/l of the peptide; however, the activity was low. The cultures showed an activity of 2·5 AU/ml. The specific activity of the purified peptide was 2 AU/mg, which is three orders of magnitude less than what is seen for bacteriocins from LAB and propionicin T1 (Deegan et al. 2006; De Vuyst & Leroy, 2007).

Gollop & Lindner (1998) investigated the mode of action of propionicin PLG-1 on different bacterial strains. Most LAB bacteriocins studied affected efflux of potassium ions from sensitive cells in surrounding medium. Propionicin PLG-1,

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Table 1. The antimicrobial activity (mm) of different LAB transformants compared with *P. thoenii* P127

	LAB transformants harbouring pLEBPLG-1				
Microorganisms	*Lc. lactis* LL108	*Str. thermophilus* ATCC19258	*Lb. bulgaricus* DSM20080	*Lb. plantarum* TF103	*P. thoenii* P127
Esch. coli DH5α	9·10 ± 0·42Aa	8·20 ± 0·39Ba	7·50 ± 0·56Ca	7·30 ± 0·54CDa	4·30 ± 0·01Ea
List. monocytogenes 18116	7·30 ± 0·22Ab	7·20 ± 0·26ABb	6·70 ± 0·25Cab	6·00 ± 0·23Db	3·20 ± 0·02Eb
Sal. enterica 25566	6·50 ± 0·26Ac	6·30 ± 0·22ABc	5·70 ± 0·23Cc	4·90 ± 0·21Dc	2·20 ± 0·01Ec

Data are expressed as mean ± SD. Means in the same column with different lowercase letters are significantly different ($P \leq 0{\cdot}05$). Means in the same row with different capital letters are significantly different ($P \leq 0{\cdot}05$)

however, did not affect efflux of potassium ions or UV absorbing material from the sensitive *Lactobacillus* strain, whereas nisin did. The authors concluded that propionicin PLG-1 has a mode of action previously not seen with other bacteriocins. It was shown that the bacteriocin interfered with the glycolysis of the target organism, and it was suggested that the bactericidal effect could be ascribed to inhibition of metabolism. Strain P127 could produce propionicin PLG-1 in skim milk. Psychrotrophic spoilage or pathogenic food-borne microorganisms such as *Listeria monocytogenes, Pseudomonas fluorescens, Vibrio parahaemolyticus* and *Yersinia enterocolitica* were killed or inhibited when inoculated into skim milk fermented by strain P127 and incubated at 10 °C. A mutant of strain P127 that did not produce propionicin PLG-1 did not affect the growth of the spoilage or pathogenic strains.

The greatest advantage of propionicin PLG-1 is to inhibit or kill the growth of the above mentioned food-borne pathogens. Therefore, it was suggested that propionicin PLG-1 has a potential use as a food preservative since it was stable after long-term storage in a dry or frozen state and rapidly kills sensitive cells upon exposure in culture medium or skim milk (Lyon et al. 1993; Hsieh & Glatz, 1996). Furthermore, a novel method for propionicin PLG-1 detection was described (Leversee & Glatz, 2001). Such method that was based on the construction of polyvalent anti-PLG-1 antiserum allowed the detection of propionicin PLG-1 precisely even on the µg-scale. Gollop et al. (2003) modified the growth medium of *P. thoenii* P127, allowing a high production system of the antibacterial peptide PLG-1. Tawfik et al. (2004) used *P. thoenii* P127 metabolites to preserve Domiati cheese.

Although intensive studies have been performed on propionicin PLG-1 chemical structure and antimicrobial activity (Lyon & Glatz, 1991, 1993; Paik & Glatz, 1995; Thierry et al. 2011), nothing was reported about its gene sequence and its molecular characterisation. Thus, we have reported for the first time *plg*-1 gene sequence and investigated its DNA alignment with other studied propionibacteria bacteriocin genes. Furthermore, we have extended propionicin PLG-1 antimicrobial spectrum by screening its activity on some common food-borne pathogens present in our climate (*Escherichia coli* DH5α, *List. monocytogenes* 18116, and *Salmonella enterica* 25566) in order to know if this bacteriocin has antimicrobial activity on these strains. Finally, the problem of propionicin PLG-1 production by very small amounts was resolved by cloning *plg-1* gene expressing propionicin PLG-1 into different dairy starters to find the most promising LAB that could be used to produce propionicin PLG-1 in sufficient amounts for further fermentation studies.

Materials and methods

Microbial strains, plasmids and growth conditions

All microbial strains used in this study and their media are listed in Table 1. *Esch. coli* DH5α was grown in LB (Luria Bertani) medium at 37 °C (Green & Sambrook, 2012). *List. monocytogenes* 18116 and *Sal. enterica* 25566 were grown in BHI (Brain Heart Infusion) medium at 37 °C according to manufacturer's instructions (Difco, 2012). Lactobacilli strains were grown in MRS medium (de Man et al. 1960), whereas lactococcal strains were grown in M17 medium (Oxoid, UK) containing 5 g/kg glucose (GM17) (Takala & Saris, 2002). *Lactococcus lactis* MG1614 harbouring the vector pLEB590 was grown in GM17 containing nisin (60 IU/ml) (Takala & Saris, 2002). All lactococcal and lactobacilli strains were grown at 30 °C. All propionibacteria strains were grown on mNLB (Modified Sodium Lactate) medium (Gollop et al. 2003).

Genomic DNA isolation, extraction from gel, primers designing, and specific PCR

Genomic DNA was isolated using Fermentas® genomic DNA purification kit, whereas DNA bands were extracted from gel (Fig. 1) using AxyPrepÔ DNA Gel Extraction Kit. The sequence of the structural gene (*plg-1*) was obtained by PCR using degenerate primers based on the N-terminal amino acid sequence, followed by a primer-walking strategy. Final primers (PLG1-F, GGATCCAATGTCGATGCC AG and PLG1-R, TGGGGTCGAGTTGCAGACCCCAAT) were designed using Geneious® software (v. 4·0·2) to isolate the complete sequence of *plg-1* gene of *P. thoenii* P127. PCR experiments were carried out with a Thermal Cycler (Techne, UK); Gold Master Mix Beads were used

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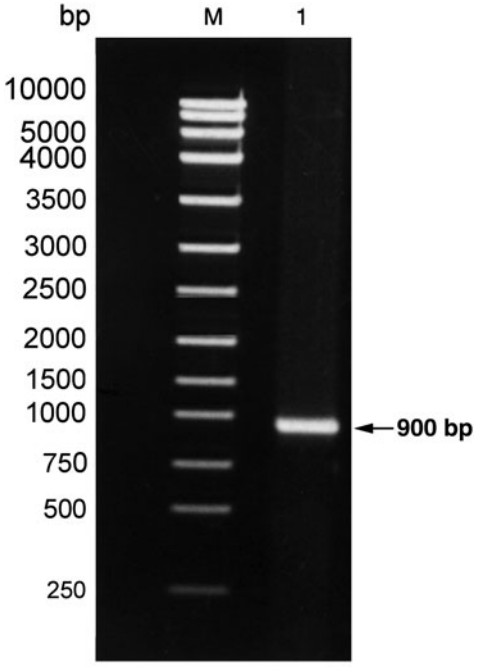


Fig. 1 Isolation of PCR product containing *plg-1* gene using the most convenient specific primers (900 bp, lane 2) beside the 1Kb DNA ladder (Fermentas, EU) (lane 1)

as recommended by the manufacturer (Bioron, Germany). The reactions (volume, 20 µl) were performed with 50 pmol of each primer. The PCR conditions used for amplification of DNA fragments containing the *plg-1* gene included a hot start at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and polymerisation at 72 °C for 6 min.

DNA manipulations

Isolation of plasmids from *Esch. coli* was carried out using BioBasic® EZ-10 plasmid spin column kit, whereas isolation of plasmids from LAB was performed using a miniprep isolation method (Shareck et al. 2004). Purification of DNA samples was performed using Fermentas® DNA purification spin column kit. DNA digestions, DNA ligations, and electroporation of *Esch. coli* strains were carried out according to Green and Sambrook (2012), while electroporation of lactobacilli strains was carried out according to Kim et al. (2005) and electroporation of lactococcal strains was performed according to Pontes et al. (2011).

Nucleotide sequence accession number

The PCR products were sequenced using ABI Prism 377 DNA sequencer (Perkin-Elmer, USA). The DNA sequence described here has been deposited in the European Nucleotide Archive under accession number HE814565.

Bacteriocin assay

Antimicrobial activity was determined by a microtitre plate assay (Holo et al. 1991). Each well of the microtitre plate contained 50 µl of two fold serial dilutions in LB or GM17 of the bacteriocin samples and 150 µl of a 100-fold diluted fresh overnight culture of the indicator strain. The plates were incubated at 30 °C for 24 to 48 h, and growth inhibition of the indicator organisms was measured spectrophotometrically at 620 nm with a microtitre plate reader. One bacteriocin unit (BU) was defined as the amount of bacteriocin that inhibited the growth of the indicator organism by 50% compared with a control culture without bacteriocin.

Data analysis

This study aimed to evaluate the capacity of propionicin PLG-1 expression by LAB transformants. Analysis of variance was carried out on all measured parameters among the control (*P. thoenii* P127, the natural producer of propionicin PLG-1) and LAB transformants to determine any significant differences during propionicin PLG-1 expression. The study was repeated in triplicate and five strains were used for each replicate ($n_{total} = 15$ and $n_{total\ for\ each\ treatment} = 5$). Statistical procedures were performed using LSMPROG GLM of the SAS program (SAS Institute, Cary, NC, USA). Statistical significance was defined at $P \leq 0{\cdot}05$.

Results

Isolation of plg-1 gene of P. thoenii P127 using degenerate PCR primers and sequencing of the PCR product

It was reported that the N-terminus of the bacteriocin PLG-1 is NVDARRARAP (Paik & Glatz, 1995; Ben-Shushan et al. 2003). Therefore, such protein sequence was translated to its convenient DNA sequence using (ExPASy) SIB Bioinformatics Resource online tool (http://www.expasy.org) and a DNA alignment between our different sequences and the reference sequence was performed using the online website EMBL-EBI ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to find the DNA sequence that is most homologous to *plg-1* gene N-terminus.

The relationship between different fully sequenced propionicins

Since this is the first paper to describe *plg*-1 gene sequence, a relationship between different fully sequenced propionicins was performed. Figure 2 shows that the sequence of PLG-1 is composed of an open reading frame of 318 nucleotides (99 aa). This data was obtained using the NCBI ORF finder website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acids sequence was further analysed using the web-based program ProtScale (http://web.expasy.org/protscale/) and the web-based program ProtParam (http://web.expasy.org/protparam). Table 2 reports the

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Fig. 2. Nucleotides sequence and the amino acids sequence of *plg-1* gene. The red capital letters represent sequence homologies to that of Ben-Shushan et al. (2003)

Table 2. The relationship between different propionicins PT1, PLG-1, PAMP, GBZ, and PCTF

SeqA	Name	Length	SeqB	Name	Length	Score
1	PT1	2210	2	PAMP	1700	61·00 ± 0·43a
1	PT1	2210	3	PCTF	347	82·00 ± 0·22b
1	PT1	2210	4	GBZ	675	76·00 ± 0·34c
1	PT1	2210	5	PLG-1	882	63·00 ± 0·48d
2	PAMP	1700	3	PCTF	347	85·00 ± 0·25e
2	PAMP	1700	4	GBZ	675	99·00 ± 0·01f
2	PAMP	1700	5	PLG-1	882	53·00 ± 0·55g
3	PCTF	347	4	GBZ	675	65·00 ± 0·44h
3	PCTF	347	5	PLG-1	882	66·00 ± 0·45i
4	GBZ	675	5	PLG-1	882	30·00 ± 0·69j

Data are expressed as mean ± SD. Means in the Score column with different lowercase letters are significantly different ($P \leq 0{\cdot}05$)

sequence homologies of different propionicins in score numbers.

Propionicin PLG-1 is well known to contain 99 amino acid residues with a MW of about 10 000 Da (10283·9 Da). Paik & Glatz (1995) have previously sequenced the N-terminus of propionicin PLG-1 and calculated its MW to be 9328. Their calculated MW agrees closely with that of 9690, determined on PAGE. The predicted amino acid composition result was similar to that of Paik & Glatz (1995) with minor differences. Neutral (Gly) and hydrophobic (Ala, Ile, Leu, Val and Pro) residues make up 20·1 and 42·4%, respectively. Such results were similar to that of Paik & Glatz (1995); 20 and 42%. Propionicin PLG-1 is predicted to contain two amino acid residues of tryptophan. Paik & Glatz (1995) noted that tryptophan might be destroyed by the acid hydrolysis method employed.

Cloning and expression of plg-1 gene of P. thoenii P127 into LAB

Propionicin PLG-1 gene (*plg-1*) was cloned into pLEB590 and electroporated into different LAB strains. The expression vector (pLEB590) was constructed totally from lactococcal DNA to be used as a cloning vehicle for LAB (Takala & Saris, 2002). Such vector fulfilled all necessary requirements to be a food-grade vector; hence, it has a small size (3·1 Kb), a multiple cloning site (MCS), a nisin immunity gene (*nisI*) as a dominant food-grade selection marker, a high copy number, and a high stability for several generations. It has a potential for use in dairy processes, in order to construct improved LAB starter strains.

Electroporation of pLEBPLG-1 into LAB strains

Electroporation efficiency of different LAB strains harbouring pLEBPLG-1 was indicated. It reached to $4{\cdot}2 \times 10^9$ cfu/µg DNA for *Lactococcus lactis* LL108, while that for *Streptococcus thermophilus* ATCC19258 was found to be $4{\cdot}1 \times 10^9$ cfu/µg DNA. On the other hand, *Lactobacillus bulgaricus* DSM20080 and *Lb. plantarum* TF103 recorded $5{\cdot}1 \times 10^8$ and $5{\cdot}2 \times 10^8$ cfu/µg DNA, respectively.

Screening for the antimicrobial activity of LAB transformants against Esch. coli DH5α, List. monocytogenes 18116, and Sal. enterica 25566

The inhibitory activity was detected in liquid culture, and maximum antimicrobial activity was found in the log and

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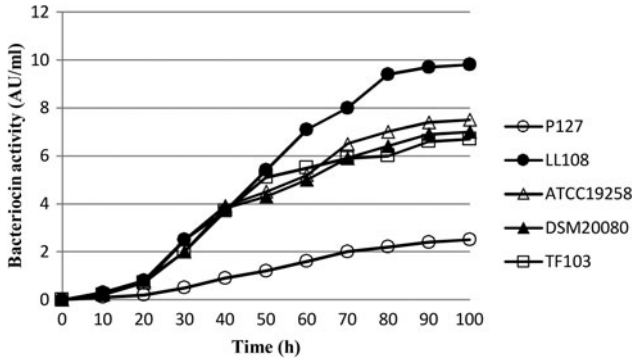


Fig. 3. Bacteriocin activities (AU/ml) of *Lc. lactis* LL108 (black circle), *Str. thermophilus* ATCC19258 (white triangle), *Lb. bulgaricus* DSM20080 (black triangle), and *Lb. plantarum* TF103 (white square) comparing with the bacteriocin activity of *P. thoenii* P127 (white circle)

early stationary growth phases of LAB transformants and *P. thoenii* P127, respectively (Fig. 3). Among the investigated microorganisms, *Esch. coli* DH5α was the most sensitive on agar plates as well as in the microtitre plate assay.

Propionicin PLG-1 has strong antimicrobial activity against the examined pathogens with special emphasis on *Esch. coli* DH5α that was the most sensitive strain, while Table 1 indicates such antimicrobial activity by inhibition zone diameters (mm) compared with the natural propionicin PLG-1 producer (*P. thoenii* P127). Thus, propionicin PLG-1 was successfully over-expressed using pLEB590 as a multi-copy expression vector.

Discussion

Several research articles have been published on the characterisation and proteomics of propionicin PLG-1 but none of them investigated its genetic characterisation in depth. For example, Hsieh & Glatz (1996) examined the long-term storage stability of propionicin PLG-1 and recommended it as a food preservative. Accordingly, Paik & Glatz (1997) enhanced propionicin PLG-1 production using fed-batch fermentation. Gollop et al. (2003) developed an efficient system for the antibacterial peptide PLG-1 production in order to be used as a natural preservative in food industries. On that basis, Tawfik et al. (2004) used the *P. thoenii* P127 whole crude metabolites to preserve Domiati cheese and named such crude extract as Propiogard. Such product proved to prolong Domiati cheese shelf life regardless of the deviations occurring in its specifications (Codex, 2012). Therefore, it was aim to focus on *plg*-1 gene cloning and expression in LAB.

After the analysis of DNA sequences of different propionicin genes using DNA alignment tools, it was noticed that *pam*A gene of *P. jensenii* LMG3032 and *gbz-1* gene of *P. thoenii* P127 are very close to each other (score = 99·0), while *plg-1* gene of *P. thoenii* P127 does not share significant homologies with other genes. Paik & Glatz (1995) indicated previously that propionicin PLG-1 seems to be different from other reported LAB bacteriocins and this indication remains true. Such observation suggests that PLG-1 bacteriocin has a different mode of action than other well-known bacteriocins. However, *pam*A, *gbz-1* and *plg-1* genes have significant sequence homologies only in their N-terminus (ARAPH). This means that the latter three genes have the same leader signal peptide (ARAPH) which direct the newly synthesised bacteriocins to be extracellular secreted through the secretory protein-conducting channel present in the plasma membrane.

Propionicin PLG-1 belongs to class II bacteriocins according to the classification of Riley & Chavan (2007). Its MW is about 10 000 Da with no modified amino acids. Propionicin PLG-1 does not belong to class IIa even its positive antilisterial activity because it does not share homology with the N-terminal consensus sequence (Tyr-Gly-Asn-Gly-Val-Xaa-Cys) across that group and it does not belong to class IIb also because it is not composed of two peptides. Class IIc includes all class II bacteriocins that do not fall into class IIa or IIb. This group is divided into two sub-groups: bacteriocins containing one or two cysteine residues or bacteriocins without cysteines. Propionicin PLG-1 does not fall into either of these sub-groups as it contains five cysteine residues. This is similar to the findings of Ben-Shushan et al. (2003) for the bacteriocin GBZ-1 classification. Through some bioinformatics analysis, propionicin PLG-1 was found to have an isoelectric point (pI) of 9·01 (using ProtParam) and a net charge of +4 (using ProtScale). We have noticed that such bacteriocin has worked efficiently in a wide range of pH (from 3 to 8; data not shown) and consequently it could be used as a food additive without the fear of losing its activity due to any normal changes in pH.

It was noted that LAB transformants over-expressed propionicin PLG-1 about three orders of magnitude more that the natural producer (*P. thoenii* P127). Such result was expected since pLEB590 vector has a strong LAB promoter (P45) that affected *plg*-1 transcription and further translation to give significant amounts of propionicin PLG-1 from the LAB transformants to inhibit the examined food-borne pathogens more efficiently than *P. thoenii* P127 (Table 1). Furthermore, it was noticed that among the LAB transformants, *Lc. lactis* LL108 (harbouring pLEBPLG-1) was the strain that most over-expressed propionicin PLG-1 (9·8 AU/ml). This could be explained since pLEB590 was constructed totally from lactococcal DNA, thus it worked more efficiently in lactococci.

The modified LAB transformants have the ability to produce such bacteriocin during its growth in dairy products and prolong their shelf life without any change of the final food product specifications including colour, taste, and texture. Therefore, they can be used as promising dairy starter cultures with dual-purpose to decrease the chances of dairy products contamination with food-borne pathogens especially where warm humid climates prevail.

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Furthermore, the most efficient LAB strain (*Lc. lactis* LL108) will be used for further fermentation studies with some optimisation steps to produce propionicin PLG-1 with sufficient amounts to be added as a promising food additive to prolong the shelf life of different dairy products.

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