Cloning and characterization of mersacidin like bacteriocin from *Bacillus licheniformis* MKU3 in *Escherichia coli*

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Abstract A putative gene encoding mersacidin like lantibiotic bacteriocin (lanA) was identified in *Bacillus licheniformis* genome. The *lanA* open reading frame codes for 74 amino acids with calculated isoelectric point of 6.7 and molecular mass of 8.2 kDa. The *lanA* gene was amplified from *B. licheniformis* MKU3, cloned in pQE30 vector and overexpressed in *Escherichia coli* M15. The recombinant peptide was purified to homogeneity using Ni-NTA chromatography and the SDS-PAGE analysis of the purified peptide revealed it to be a monomer with molecular mass of ~8.5 kDa. The purified bacteriocin showed wide spectrum activity against gram-positive pathogens. The peptide was found to be stable under in wide range of pH, temperature tolerant and resistant to the proteolytic enzymes. The stable nature of the bacteriocin to high temperature and resistant to various chemicals it also exhibited antimicrobial activity against food-borne pathogens make this bacteriocin as potent attractive antimicrobial agent in food products.

Keywords *Bacillus licheniformis*, bacteriocin, mersacidin · Food-borne pathogens · Heat stable peptide · Protease resistant

Introduction

Bacteriocins are ribosomally synthesized peptides that affect the growth and viability of closely related microorganisms. Earlier studied bacteriocins are colicins produced by the Enterobacteriaceae (Pugsley 1984) and antimicrobial peptides produced by several Gram-positive bacteria. In particular, bacteriocins produced by lactic acid bacteria have gained interest due to their potential application as natural food preservatives and therapeutic agents (Tagg et al. 1976). The bacteriocins are grouped into four distinct classes (Klaenhammer 1993) namely the class I bacteriocins are lantibiotics type containing unusual thioether amino acids, which are generated through post-translational modification (McAuliffe et al. 2001). The class II bacteriocins are small (<10 kDa), heat-stable, membrane-active peptides (Nes and Holo 2000) which are further divided into three subgroups: IIA are *Listeria*-active peptides containing N-terminal consensus sequence YGNGVXC (where X is any amino acid); IIB peptides are poration complexes that require two different peptides for its activity; and IIC are extracellular peptides dependent on the general secretory (sec) pathway. The class III bacteriocins are large (>30 kDa), heat labile proteins whereas the class IV bacteriocins contain essential lipid or carbohydrate moieties.

Similar to Lactic acid bacteria, several *Bacillus* spp., such as *B. subtilis* and *B. licheniformis*, are ‘generally recognized as safe’ (GRAS) bacteria (Sharp et al. 1989). Antimicrobial peptides or lantibiotic bacteriocins are produced by many gram positive bacteria including *B. subtilis*, *B. thuringiensis*, *B. stearothermophilus* (Sharp et al. 1979), *B. licheniformis* (Jamal et al. 2006), *B. megaterium* (Kiss et al. 2008), *B. thermoleovorans* (Novotny and Perry 1992), *B. cereus* (Bizani et al. 2008) and *B. coagulans* (Hyronimus et al.

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1998). Several of the above mentioned bacteriocins were not purified to homogeneity for the study of biochemical characterizations and genetic information on these peptides are limited. The well characterized bacteriocins are subtilin, subtilisin from *Bacillus subtilis* (Shelburne et al. 2007) and megacin from *B. megaterium* (Kiss et al. 2008). Since several bacterial genome sequences are available in public databases, mining novel bacteriocins may be an attractive alternative. These bacteriocins could be used as an effective preservative to control the food-borne pathogens. The recombinant cells would merit consideration as an alternative experimental model for the heterologous production and functional expression of bacteriocin, the ability to grow in minimally defined media, as well as for the fast and efficient recovery of the bacteriocin from the supernatant of the recombinant producer.

Since there is no report on cloning of lantibiotic mersacidin gene from *B. licheniformis*, an attempt was made to clone and express this gene. In the present study, our objective was to identify and characterize a bacteriocin which is having wide spectrum activity against several food borne pathogens.

Materials and methods

Bacterial strains and growth conditions

Bacillus licheniformis (MKU3) from our laboratory stock (Microbial Type Culture Collection, Chandigarh, India; MTCC 5271) was grown in nutrient broth (Hi Media, Mumbai, India) at 37 °C for 18 h. *E. coli* M15 [pRep4] (Qiagen, Hilden Germany) was grown in Luria-Bertani (LB) medium (Hi Media, Mumbai, India) at 37 °C for 12 h in a shaking incubator. All indicator bacterial strains and their growth media used are listed in Table 3.

Cloning

Total genomic DNA from *B. licheniformis* MKU3 was isolated using the Qiagen DNA Purification Kit (Qiagen, Germany) and used as target DNA for PCR amplification of the gene *lanA* (fragment P, 216 bp) with primers *lanAF* (5'-GCTACATATGTCAAAAAGGAAATGATTC - 3') and *lanAR* (5'-ATACTCGACGTTACAGCTTGGCATGC - 3'). The primer were designed using the sequence available in the nucleotide accession number (CP000002; AE001733) by Netprimer software (www.premierbiosoft.com). PCR amplifications were performed in 50- μ l reaction mixtures using 3 μ l of purified DNA, 70 pmol of each primer and 1 U of Biotools DNA polymerase (Biotools, Madrid, Spain). Samples were subjected to an initial cycle of denaturation (95 °C for 2 min), followed by 35 cycles of denaturation (94 °C for 45 s), annealing (64 °C for 30 s) and elongation (72 °C for 30 s to 2 min), ending with a extension at 72 °C for

5 min, in a DNA thermal cycler (Biorad; Techne, Cambridge, UK). PCR-generated fragments were purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) before cloning into pQE-30UA vector (Qiagen, Hilden, Germany) and the construct pQLAN was used transform *E. coli* M15 [pRep4] by using Bio-Rad gene pulser electroporation unit (Bio-Rad Laboratories, Richmond, California).

Restriction digestion of DNA

An aliquot of DNA sample was mixed with 2 μ l of appropriate 10X restriction buffer and the volume made up to 20 μ l with MQ water. The reaction was started with the addition of the enzyme (*EcoRI* and *HindIII*) and incubated at 37 °C/30 °C for 2 h. Incubation at 70 °C for 3 min terminated the reaction. The digested DNA was observed on 1 % agarose gel. The sizes of the restriction fragments were determined by comparing their mobility with appropriate molecular weight DNA markers.

Ligation

A 224-bp fragment of the bacteriocin gene (*lanA*) was amplified by PCR and ligated into the *EcoRI* and *HindIII* site of pQE-30UA to form the plasmid pQELanA.

Transformation of *E. coli*

E. coli was transformed with plasmid DNA according to CaCl₂ method (Maniatis 1982). To 100 μ l of competent cells, ~100 ng of plasmid DNA in ligation mixture was added. This was kept on ice for 45 min. A heat shock at 42 °C was given for 90 s. The cells were immediately chilled on ice for about 10 min. To this mix 900 μ l of LB broth was added and the cells were incubated at 37 °C (60 min) for expression and plated on LB agar supplemented with X-gal (20 mg l⁻¹), IPTG (40 mg l⁻¹) or appropriate antibiotics. After overnight incubation at 37 °C, all white colonies were scored as transformants and then screened for the selected marker or activity (Maniatis 1982).

Plasmid isolation

The plasmid DNA was isolated by the method of Birnboim and Doly, (1979). Late log phase *E. coli* cultures (1.5 ml) were transferred to a microcentrifuge tubes and centrifuged at 6,000 rpm for 10 min. The cells were resuspended completely in 150 μ l of TEG buffer (Tris HCl 25 mM, EDTA 10 mM, glucose 50 mM pH 8.0). The cells were lysed, thereafter, by the addition of 300 μ l of lysis mix (NaOH 0.2 N, SDS 1 % w v⁻¹), mixed thoroughly by inverting the tubes 4–6 times, and incubated for 5 min in room

temperature. After the addition of 225 μl of 3 M potassium acetate buffer (pH 4.8), the tubes were gently mixed by inverting the tubes 5–6 times, incubated on ice for 30 min and centrifuged at 4 °C for 15 min (13,000 rpm). To the supernatant, 375 μl of isopropanol was added and the tubes were kept at room temperature for 15 min and the nucleic acid precipitate was collected by centrifugation at 10,000 rpm for 10 min. The DNA pellet was resuspended in 20 μl of TE (Tris HCl 10 mM, EDTA 1 mM).

Expression and purification of bacteriocin

E. coli M15 harboring recombinant plasmids were grown upto 0.6 OD at 600 nm in LB medium (g L⁻¹): tryptone - 10.0; yeast extract - 5.0; NaCl - 10.0 supplemented with 30 $\mu\text{g ml}^{-1}$ kanamycin at 37 °C. The cells were induced by adding 400 $\mu\text{mol L}^{-1}$ concentrations of IPTG and incubated at 37 °C for an additional 4 h. The cell pellets were resuspended in 20-mM sodium phosphate buffer (pH 7.5) in the presence of 1 mg ml⁻¹ lysozyme and incubated on ice for 30 min. Cells were disrupted by sonication at 4 °C for 5 min, and the lysate was centrifuged at 14,000 $\times g$ for 20 min at 4 °C to remove the cell debris. The resulted crude extract was concentrated by ultrafiltration (MW cutoff, 10 kDa, Amersham pharmacia, Uppsala, Sweden) and further subjected to purification. The fraction was further subjected to previously equilibrated with a binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) a Ni-NTA superflow column (5 $\times$ 1 cm, Amersham Pharmacia, Uppsala, Sweden). Unbounded proteins were washed out with a washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). The recombinant protein was eluted from the column with an elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The fractions were assayed for bacteriocin activity and proteins were resolved on SDS-PAGE.

SDS-PAGE analysis of purified bacteriocin

Proteins were separated by Tricine-SDS-PAGE as described by Schägger and Von Jagow (1987). Molecular-weight-marker proteins with sizes ranging from 3 to 47 kDa (Bangalore Genei, Bangalore, India) were used. The gels were fixed and one-half was stained with Coomassie Blue R250, and the position of the active bacteriocin was determined on an unstained gel. *Kurthia gibsonii* GCS6 (10⁶ CFU/ml) is the indicator organism, suspended in nutrient agar (0.8 %) (Hi-Media, Mumbai, India) was used to overlay the gel to detect the zone of clearance after overnight incubation at 37 °C (Ivanova et al. 1998).

Bacteriocin assay and mode of action

Bacteriocin activity was determined by using agar well diffusion assay. Indicator bacteria were inoculated as lawn on the nutrient agar and purified bacteriocin was added into the wells in the lawn. The plates were incubated for overnight at 37 °C and diameter of inhibition zones was measured. Unit of antimicrobial activity (AU) was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition (Green et al. 1997). Specific activity of bacteriocin was expressed as units per mg of protein (AU mg⁻¹). Protein concentration was estimated by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

The antimicrobial protein (50 AU ml⁻¹) was added to exponential growing cells of *K. gibsonii* GCS6 in nutrient medium. Sterile nutrient medium was used as control. Changes in the turbidity of the cultures were recorded at an OD of 600 nm and the number of colony-forming units (CFU) was determined by plating the samples on nutrient agar.

Effects of pH, temperature, chemicals and proteases

Aliquot of the purified bacteriocin was treated with proteinase K, trypsin or pronase E (2 and 10 mg ml⁻¹) (Sigma, St Louis, USA) at 37 °C for 1 h against *Kurthia gibsonii* GCS6. The set of aliquots of purified bacteriocin was kept at temperatures ranging from 30 to 100 °C for 30 min, 100 °C for 5–60 min and 121 °C for 15 min. To analyze the pH stability, the purified bacteriocin were incubated at different sodium phosphate buffers pH levels ranging from 3.0 to 10.0 for 2 h at 25 °C. Later neutralized to pH 7.0 and then tested for antimicrobial activity. Another set of samples were incubated with the selected chemicals for 1 h at 37 °C. Organic solvents were used at the working concentration of 50 % (v v⁻¹). The detergents Tween 20 and 80 (10 % v v⁻¹), sodium deoxycholate (0.1 % w v⁻¹), EDTA and trichloroacetic acid (10 mM and 0.61 M) concentrations were used. After treatment with TCA, samples were centrifuged at 10 000 g for 5 min and the supernatant was neutralized to pH 7.0. In all experiments, untreated purified bacteriocin was served as control.

Homology modeling

The Deep View programme (version 3.7) in SWISS-MODEL server was used to generate the 3D model of bacteriocin. Using the integrated sequence alignment tools and structural superimposition algorithms, the

Fig. 1 Cloning of *lanA* gene in pQE30A **a** PCR amplification of Lantibiotic mesarcidin gene *lanA* Lane M- 100 bp DNA Marker; Lane 2- PCR amplicon of *B. licheniformis* MKU3 genomic DNA with *lanA* specific primers. **b** Restriction digestion of plasmid pQLAN. Recombinant plasmid pQLAN was isolated and digested with *EcoRV* and *HindIII* and resolved on 0.7 % agarose gel. Lane M- 1 kb DNA Marker; Lane 1- Plasmid pQLAN from recombinant *E. coli*; Lane 2- *EcoRV* and *HindIII* digested pQLAN; Lane 3- Amplicon obtained from the PCR with *lanA* gene specific primers

target sequence was mapped on the modeling templates. The sequence alignment was then optimized manually and the resulting model of *B. licheniformis* bacteriocin

was analyzed using Swiss-PDB Viewer. Figures were created with PyMOL version 0.98. The model was validated using WHATCHECK programme available at the WHAT IF web interface (Rodriguez et al. 1998).

Results

Cloning and sequencing of bacteriocin gene of *B. licheniformis* MKU3

The whole genome of *B. licheniformis* ATCC 14580 sequence analysis revealed the presence of an ORF (224 bp) that code for putative mesarcidin like lantibiotic precursor. This ORF was amplified from genomic DNA of *B. licheniformis* MKU3 and directly cloned into pQE30-UA vector and expressed in *E. coli* M15[pREP4], the recombinant plasmid was designated as pQLAN (Fig. 1a). The size of the cloned gene in the recombinant plasmid was confirmed by digesting with restriction enzymes *EcoRV* and *HindIII* by electrophoresis (Fig. 1b). Nucleotide and deduced amino acid sequence of putative

Fig. 2 a Nucleotide and deduced amino acid sequence of *lanA* of *B. licheniformis* MKU3 **b** Phylogenetic analysis of bacteriocin. The amino acid sequence of bacteriocin LanA was used to analyze the similarity relationship with related lantibiotics. The dendrogram was generated with ClustalX in Phylip format using the neighbor-joining method and displayed in Treeview

Table 1 Similarity index of LanA of *B. licheniformis* MKU3 with other mersacidins from related bacteria

Strains	<i>B. licheniformis</i> MKU3	<i>B. licheniformis</i> ATCC 14580	<i>B. holodurans</i>	<i>R. gnavus</i>	<i>S. ratti</i>	<i>S. mutans</i>
<i>B. licheniformis</i> MKU3	–	100	60.52	45.94	43.47	44.28
<i>B. licheniformis</i> ATCC 14580	100	–	60.52	45.94	43.47	44.28
<i>B. holodurans</i>	43.42	43.42	–	53.75	48.64	49.33
<i>R. gnavus</i>	29.72	29.72	32.50	–	35.82	35.29
<i>S. ratti</i>	33.33	33.33	36.48	17.91	–	98.38
<i>S. mutans</i>	32.85	32.85	36.00	17.64	96.77	–

The percentage identity of amino acid sequences are listed in lower diagonal and the percentage similarity are listed in upper diagonal

bacteriocin *lanA* of *B. licheniformis* MKU3 is presented in Fig. 2a and b. BlastN analysis of the *lanA* sequence showed 100 % identity with lantibiotic mersacidin precursor of *B. licheniformis* 14580/DSM13. The sequence analysis of the cloned *lanA* revealed that it encodes a polypeptide of 74 amino acids, with no signal peptide and a calculated molecular mass of 8.2 kDa and isoelectric point (pI) of 6.7. The deduced amino acid sequence of *lanA* gene showed 100, 60.52, 45.94, 43.47, and 44.28 % sequence identity with mersacidin from *B. licheniformis* DSM13, *Bacillus holodurans*, *Ruminococcus gnavus*, *Streptococcus ratti* and *Streptococcus mutans* respectively (Table 1). The secondary structure of *lanA* peptide is composed of 47.30 % of random coil, 32.43 % of alpha

helix, 13.51 % of extended strand and 6.76 % of beta turn.

Expression of *lanA* and molecular weight determination

The plasmid pQLAN carrying *lanA* expressed in *E. coli* M15 [pREP4] using the T5 promoter. Upon induction with IPTG (0.5 mM), the *lanA* gene was expressed in *E. coli* M15 (pQLAN) as revealed from the SDS-PAGE analysis. The bacteriocin was purified through nickel-nitrilotriacetic acid Ni-NTA affinity chromatography (Fig. 3). The expected molecular mass of ~8.5 kDa was detected from *E. coli* M15 (pQLAN) and result also well agreed with the predicted molecular mass for the bacteriocin. In parallel, zymogram analysis of LanA with *K. gibsonii* GCS6 as an indicator revealed an inhibition zone at the position of ~8.5 kDa protein band (Fig. 4). This result suggested that the bacteriocin *lanA* is a monomer with molecular weight of ~8.5 kDa. The specific

Fig. 3 Affinity chromatographic purification of recombinant bacteriocin LanA from *E. coli* M15 (pQLAN). Purification was performed at 22 °C in FPLC using HiTrap (5 ml) metal chelating column (Amersham Pharmacia Biotech, USA). Clear cell lysate (50 ml) was passed through the column at a flow rate of 0.5 ml min⁻¹ and washed with 10 column volumes of start buffer (20 mM sodium phosphate (pH 7.0), 100 mM NaCl and 20 mM imidazole). His-tagged protein was eluted with imidazole gradient using the elution buffer (20 mM sodium phosphate (pH 7.0), 100 mM NaCl and 400 mM imidazole). Fractions (2 ml) were collected, assayed for bacteriocin activity and the active fractions were pooled

Fig. 4 SDS-PAGE and zymogram analyses of purified bacteriocin. Proteins were resolved on a 15 % Tricine SDS-PAGE and stained with Coomassie brilliant blue R-250. Zymogram analysis was performed by overlaying the 0.7 % soft agar containing 10⁶ indicator strain *K. gibsonii* GCS6. Lane M-Molecular weight protein markers; Lane 1- Cell extract of *E. coli* (pQLAN) induced with 0.4 mM IPTG; Lane 2-Cell extract of *E. coli* M15; Lane 3-LanA protein of *E. coli* M15 (pQLAN) purified through affinity chromatography; Lane 4- purified LanA showing clear zone of inhibition on direct overlay of indicator strain *K. gibsonii* GCS6 on the gel

Table 2 Purification of LanA from *E. coli* M15 (pQLAN)

Purification step	Volume (ml)	Total activity (AU)	Specific activity (AU mg ⁻¹)	Purification fold	Recovery (%)
Cell lysate	150	2,450	1,700	1	100
Ultrafiltration	50	1,800	4,100	2.4	73.5
Affinity Chromotography	10	250	49,000	29	10.2

activity of purified LanA was 49×10^2 AU ml⁻¹ and thus resulting in purification fold of 29 (Table 2).

Characterization of purified bacteriocin LanA

Bacteriocin assay

The antibacterial spectrum of purified of bacteriocin LanA was determined by agar well diffusion method. The purified LanA exhibited the broad spectrum of antagonistic activity against Gram-positive bacteria such as *Bacillus* sp., *Lactobacillus*, *Staphylococcus* sp. and *Streptococcus* sp. however not against Gram negative bacteria (Table 3). To determine lethal effect of LanA bacteriocin activity against sensitive strains, purified bacteriocin LanA was added to the cell suspension of *K. gibsonii* GCS6 (10^7 CFU ml⁻¹). The number of viable bacterial cells after the exposure to LanA bacteriocin rapidly decreased (Fig. 5). The reduction in

viable cell number was approximately 50 % in 5 h. These results strongly suggested that the LanA exhibits bactericidal effect on the indicator organism.

Effects of proteolytic enzymes, heat, pH and chemicals on LanA activity

The recombinant LanA against the indicator strain *K. gibsonii* GCS6 was examined for its sensitivity to several proteases, chemicals, heat and pH and the results are summarized in Table 4. The purified LanA was resistant to proteinase K, pronase E and trypsin treatment. The LanA was stable to heat treatment up to 100 °C but unstable at 121 °C for 15 min and showed stability for wide range of pH 3–10. The LanA bacteriocin was also stable to various chemicals such as TCA, chloroform, methanol, butanol, toluene, Tween- 80, EDTA and SDS.

Table 3 Antibacterial activity of the purified LanA bacteriocin

Indicator strain	Medium used	Antibacterial activity
<i>Staphylococcus aureus</i> GCS1	NA	+
<i>Bacillus cereus</i> GCS2	NA	+
<i>Bacillus cereus</i> GCS3	NA	+
<i>Staphylococcus epidermidis</i> GCS4	NA	+
<i>Staphylococcus epidermidis</i> GCS5	NA	+
<i>Kurthia gibsonii</i> GCS6	NA	+
<i>Micrococcus luteus</i> GCS7	NA	+
<i>Bacillus subtilis</i> GCS8	NA	+
<i>Streptococcus fecalis</i> GCS9	NA	+
<i>Bacillus cereus</i> GCS10	NA	+
<i>Bacillus cereus</i> GCS11	NA	+
<i>Lactobacillus acidophilus</i> GCS12	MRS	+
<i>Lactobacillus lactis</i> NRRL B-1821	MRS	+
<i>Lactobacillus acidophilus</i> NRRL B-4495	MRS	+
<i>Bacillus subtilis</i> NRRL B-4219	NA	+
<i>Staphylococcus epidermidis</i> NRRL B-4268	NA	+
<i>Bacillus smithii</i> NRS-173	NA	+
<i>Micrococcus luteus</i> NRRL B-287	NA	+
<i>Serratia marcescens</i> ATCC27117	NA	–
<i>Pseudomonas fluorescens</i> NRRL B10	NA	–
<i>Escherichia coli</i> DH5α	LB	–

The media used in the bioassay were NA: Nutrient Agar, MRS: deMan Rogosa Sharpe Agar, LB: Luria Bertani Agar, GCS1–12 Laboratory strains

Fig. 5 Effect of LanA on the viability of *K. gibsonii* GCS6 The culture of *K. gibsonii* GCS6 was incubated at 37 °C in 50 mM phosphate buffer without (◆) and with (■) 50 AU of LanA

Structure prediction by homology modeling

B. licheniformis MKU3 LanA sequence did not find any matching sequence in PDB databank. It showed only 50 % sequence similarity with KvAP potassium channel voltage sensor membrane protein. This degree of sequence similarity is sufficient to make a homology model of LanA. The Swiss-PDB Viewer (www.expasy.org/spdbv) supported by the SWISS-MODEL server was used to generate the 3D model (Fig. 6). The structure evaluation server WHAT IF (www.cmbi.kun.nl/gv/servers/WIWWWI) was used for validation of the model. The modeled structure was 85 % correct by the validation tool. The plot indicates that the backbone conformations of amino acid residues are scattered within the acceptable regions (contoured areas). The modeled structure of bacteriocin of LanA indicated that bacteriocin

Table 4 Effect of different enzymes, temperature, and pH on LanA activity against *K.gibsonii* GCS6

Treatment	Bacteriocin activity
Trypsin, 1 mg ml ⁻¹	+
Proteinase K, 1 mg ml ⁻¹	+
Pronase, 1 mg ml ⁻¹	+
20–100 °C for 10 min	+
100 °C for 10–60 min	+
121 °C/15 min	-
pH 3.0–10.0	+
Acetone (50 % v v ⁻¹)	+
n- butanol (50 % v v ⁻¹)	+
Chloroform (50 % v v ⁻¹)	+
Methanol (50 % v v ⁻¹)	+
Toluene (50 % v v ⁻¹)	+
TCA (0.6 mM)	+
Tween-80 (10 % v v ⁻¹)	+
EDTA (10 mM)	+
SDS (10 % w v ⁻¹)	+

Fig. 6 Surface and ribbon model of the predicted structure of LanA

LanA belongs to pore forming membrane and cell surface proteins.

Discussion

Genome mining approach is considered to be a valuable tool for the development of new antimicrobials. In the present study, a putative mesarcidin like bacteriocin gene (*lanA*) was identified in the whole genome sequence of *B. licheniformis* ATCC 14580. Previously Choi et al. (2001) employed nisin gene-specific primers to clone bacteriocin from *L. lactis* A164. Sakacin G (class IIa bacteriocin) *L. sake* 2512 and haloarchaeal bacteriocin (halocin H4) in *Haloferax mediterranei* R4 have been cloned by PCR based technique (Simon et al. 2002; Cheung et al. 1997).

The development of heterologous expression systems for bacteriocin production may offer several advantages over native systems. Some of the small, class II bacteriocins namely mesentericin Y105, gassericin A, pediocin PA-1, divergicin V41, enterocin A and enterocin P have been expressed in *E. coli* (Gutierrez et al. 2005; Moon et al. 2006). In this study, mesarcidin like bacteriocin *lanA* gene was cloned and expressed in *E. coli* M15 [pREP4]. The *lanA* was poorly expressed in *E. coli* and the level of bacteriocin produced in recombinant strain was 10 times lower than that of produced by the wild type strain. The overproduced proteins in *E. coli* system are often aggregated to form inclusion body as a result of their improper folding (Baneyx and Mujacic 2004). Similarly, the activity of enterolysin A was 8.5 times lower than the native bacteriocin (Nigutova et al. 2008). The low level of expression of boticin B was found to be due to the absence of additional genes for regulatory activity, immunity, peptide modification and protein translocation. *C. botulinum* 62A transconjugant carrying boticin B showed 2 mm inhibition zone smaller than that observed with the wild type *C. botulinum* 6213B culture filtrate (8 mm) (Dineen et al. 2000). Since LanA was produced as His-tagged protein in the recombinant strain, it was purified employing Ni-NTA affinity chromatography.

The antibacterial activity range of LanA is rather narrow and limited to Gram-positive bacteria such as *B. subtilis*, *B. smithii*, *B. megaterium*, *K. gibsonii*, *Staphylococcus* and *Streptococcus* sp. But in earlier reports *B. licheniformis* produced a novel protein (30.7 kDa) with antibacterial activity against methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococci* and *L. monocytogenes* (Jamal et al. 2006). Martirani et al. (2002) reported that bacillocin 490 is active principally against species phylogenetically related organisms. *B. licheniformis* P40 produced an antimicrobial peptide that showed a broad spectrum of activity against several spoilage and pathogenic microorganisms, and *Corynebacterium fimi* NCTC 7547 (Oliveira et al. 1998). Due to relative stability to pH, temperature and resistant to various chemicals and solvents, LanA was characterized as a bacteriocin-like protein (Klaenhammer 1993). Due to the resistance to proteolytic enzymes, the LanA bacteriocin is differed from other bacteriocin produced by *Bacillus* spp. (Hyronimus et al. 1998).

Three-dimensional structure of a protein provides valuable insight into the molecular basis of its function. Protein structure elucidation by X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) is still a time-consuming and expensive process. Therefore, a 3-D model of LanA bacteriocin was generated using crystal structures of KvAP potassium channel voltage sensor membrane protein as template and structure validation at the server WHAT IF (Rodriguez et al. 1998). Ramachandran plot indicated that most of the residues are scattered within the acceptable regions of allowed conformation. Voltage-dependent ion channels respond to changes in cell membrane voltage and permit the propagation of action potentials. The structures of several voltage sensor toxins have been determined (Lee et al. 2005; Takahashi et al. 2004). Typical of small disulphide core proteins are well-structured globular proteins about 35 amino acids. The voltage sensor toxins exhibit unique feature of a strong hydrophobic moment at one face. The LanA bacteriocin from the present study was also hydrophobic and interacts with membrane of the indicator strains. Previously, Sanchez-Barrena et al. (2003) has also reported that bacteriocin AS-48 is a membrane interacting peptide which displays antimicrobial spectrum against Gram-positive and Gram-negative bacteria by the accumulation of positively charged molecules at the membrane surface leading to a disruption of the membrane potential. Our study also suggests that LanA bacteriocin to be a membrane interactive peptide.

Conclusion

Over the past several years, bacteriocins have received significant attention due to their potential application in food industry to provide microbiologically safe foods. The most

attractive feature of bacteriocins is their improved potential as food preservatives when compared to conventional chemical preservatives. Although, the antimicrobial activity of bacteriocins is relatively well understood, their use as safe biopreservatives in food and feed is still limited. So far, nisin, a small antimicrobial peptide produced by *Lactococcus lactis* subsp. *lactis*, is the only bacteriocin officially approved in the EU as food additive for protection against bacterial pathogens. Necessary precondition before bacteriocins can be applied in foods and feed is assessing their antimicrobial activity and toxicity. Moreover, isolation and purification of bacteriocins from their native hosts is time-consuming and laborious process. In the present study, we have cloned and expressed bacteriocin gene in *E. coli*. As reported in our earlier study, this bacteriocin has a broad antimicrobial spectrum that inhibits many potentially pathogenic microorganisms, including food-borne pathogens. Successful expression of biologically active form of recombinant bacteriocin in the soluble fraction and their broad antimicrobial activity may facilitate high level production of this bacteriocin, purification and use as safe antimicrobial food and feed additive.

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References

- Baneyx F, Mujacic M (2004) Recombinant protein folding and misfolding in *Escherichia coli*. *Nat Biotechnol* 22:1399–1408
- Bimboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 24:1513–1523
- Bizani D, Morrissy JA, Dominguez A, Brandell A (2008) Inhibition of *Listeria monocytogenes* in dairy products using the bacteriocin-like peptide cerein 8A. *Int J Food Microbiol* 121:229–233
- Cheung J, Danna KJ, O'Connor EM, Price LB, Shand RF (1997) Isolation sequence and expression of the gene encoding halocin H4 a bacteriocin from the halophilic archaeon *Haloferax mediterranei* R4. *J Bacteriol* 179:548–551
- Choi HJ, Cheigh CI, Kim SB, Pyun YR (2001) Production of a nisin-like bacteriocin by *Lactococcus lactis* subsp. *lactis* A164 isolated from Kimchi. *J Appl Microbiol* 88:563–571
- Dineen SS, Bradshaw M, Johnson EA (2000) Cloning, nucleotide sequence, and expression of the gene encoding the bacteriocin Boticin B from *Clostridium botulinum* strain 213B. *Appl Environ Microbiol* 66:5480–5483
- Green G, Dicks LMT, Bruggeman G, Vandamme EJ, Chikindas ML (1997) Pediocin PD-1, a bactericidal antimicrobials peptide from *Pediococcus damnosus* NCFB 1832. *J Appl Microbiol* 83:127–132
- Gutierrez J, Criado R, Citti R, Martin M, Herranz C, Nes IF, Cintas LM, Hernandez PE (2005) Cloning production and functional expression of enterocin P a sec-dependent bacteriocin produced by *Enterococcus faecium* P13 in *Escherichia coli*. *Int J Food Microbiol* 103:239–250

- Hyronimus B, Le Marrec C, Urdaci MC (1998) Coagulin a bacteriocin-like inhibitory substance produced by *Bacillus coagulans* I4. *J Appl Microbiol* 85:42–50
- Ivanova I, Miteva V, Stefanova TS, Pantev A, Budakov I, Danova S, Montcheva P, Nikolova I (1998) Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. *Int J Food Microbiol* 42:147–158
- Jamal MT, Morris PC, Hansen R, Jamieson DJ, Burgess JG, Austin B (2006) Recovery and characterization of a 30.7-kDa protein from *Bacillus licheniformis* associated with inhibitory activity against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci* and *Listeria monocytogenes*. *Mar Biotechnol* 8:587–592
- Kiss A, Baliko G, Csorba A, Chuluunbaatar T, Medzihradsky KF, Alföldi L (2008) Cloning and characterization of the DNA region responsible for megacin A-216 production in *Bacillus megaterium* 216. *J Bacteriol* 190:6448–6457
- Klaenhammer T (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Lett* 12:39–86
- Lee SY, Lee A, Chen J, MacKinnon R (2005) Structure of the KvAP voltage-dependent K⁺ channel and its dependence on the lipid membrane. *PNAS* 102:15441–15446
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275
- Maniatis, Tom (1982) Molecular cloning: a laboratory manual (edited by T. Maniatis, E.F. Fritsch, J. Sambrook) pp 507–520
- Martirani L, Varcamonti M, Naclerio G, De Felice M (2002) Purification and partial characterization of bacillocin 490 a novel bacteriocin produced by a thermophilic strain of *Bacillus licheniformis*. *Microbiol Cell Fact* 1:1
- McAuliffe O, Ross RP, Hill C (2001) Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol Rev* 25:285–308
- Moon GS, Pyun YR, Kim W (2006) Expression and purification of a fusion-typed pediocin PA-1 in *Escherichia coli* and recovery of biologically active pediocin PA-1. *Int J Food Microbiol* 108:136–140
- Nes I, Holo H (2000) Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers* 55:50–61
- Nigmatova K, Serencova L, Pikhova M, Javorsky P, Pristas P (2008) Heterologous expression of functionally active enterolysin A class III bacteriocin from *Enterococcus faecalis*, in *Escherichia coli*. *Protein Expr Purif* 60:20–24
- Novotny JF, Perry JJ (1992) Characterization of bacteriocins from two strains of *Bacillus thermoleovorans*, a thermophilic hydrocarbon-utilizing species. *Appl Environ Microbiol* 58:2393–2396
- Oliveira AA, Farias LM, Nicoli JR, Costa JE, Carvalho MA (1998) Bacteriocin production by *Fusobacterium* isolates recovered from the oral cavity of human subjects with and without periodontal disease and of marmosets. *Res Microbiol* 149:585–594
- Pugsley AP (1984) The ins and outs of colicins. II. Lethal action, immunity and ecological implications. *Microbiol Sci* 1:203–205
- Rodriguez E, Martinez MI, Medina M, Hernandez PE, Rodriguez JM (1998) Detection of enterocin AS-48 producing dairy enterococci by dot-blot and colony hybridization. *J Dairy Res* 65:143–148
- Sanchez-Barrena MJ, Martinez-Ripoll M, Galvez A, Valdivia E, Maqueda M, Cruz V, Albert A (2003) Structure of bacteriocin AS-48: from soluble state to membrane bound state. *J Mol Biol* 334: 541–549
- Schagger H, Von Jagow G (1987) Tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis for the separation of protein in the range from 1 to 100 kDa. *Anal Biochem* 166:368–379
- Sharp RJ, Bingham AHA, Comer MJ, Atkinson A (1979) Partial Characterization of a Bacteriocin (Thermocin) from *Bacillus stearothermophilus* RS93. *J Gen Microbiol* 111:449–451
- Sharp RJ, Scawen MD, Atkinson T (1989) Fermentation and downstream processing of *Bacillus*. In: Harwood CR (ed) *Bacillus*. Plenum Press, New York, pp 255–292
- Shelburne CE, An FY, Dholpe V, Ramamoorthy A, Lopatin DE, Lantz MS (2007) The spectrum of antimicrobial activity of the bacteriocin subtilisin A. *J Antimicrob Chemother* 59:297–300
- Simon L, Fremaux C, Cenatiempo Y, Berjeaud JM (2002) Sakacin G, a new type of antilisterial bacteriocin. *Appl Environ Microbiol* 68: 6416–6420
- Tagg JR, Dajani AS, Wannamaker LW (1976) Bacteriocins of Gram positive bacteria. *Microbiol Rev* 40:722–756
- Takahashi SX, Miriyala J, Colecraft HM (2004) Membrane-associated guanylate kinase-like properties of β -subunits required for modulation of voltage-dependent calcium channels. *PNAS* 101:7193–7198