Laterosporulin10: a novel defensin like Class IId bacteriocin from Brevibacillus sp. strain SKDU10 with inhibitory activity against microbial pathogens

Piyush Baindara, Nisha Singh, Manish Ranjan, Nayudu Nallabelli, Vasvi Chaudhry, Geeta Lal Pathania, Nidhi Sharma, Ashwani Kumar, Prabhu B. Patil and Suresh Korpole

INTRODUCTION

Low-molecular weight antimicrobial peptides (AMPs) produced by bacteria are usually called bacteriocins. However, bacteriocins were categorized as ribosomally synthesized and post-translationally modified peptides (RiPPs) in a recent classification (Arnison et al., 2013). Bacteriocins with unique features have been reported from a multitude of bacteria that are classified into various groups based on their structure and functional characteristics (Klaenhammer, 1993). While class I bacteriocins contain antimicrobial peptides with extensive post-translational modifications, class II includes unmodified peptides which are subdivided into various subclasses. Bacteriocins are under consideration as potential alternatives to antibiotics to treat diverse pathogenic bacteria (Cotter et al., 2013), and a significant step along this direction is the exploration of the antimicrobial peptides (AMPs) for anti-mycobacterial activity (Gutsmann T, 2016). AMPs are considered important and promising antimicrobial candidates (Semvua et al., 2015) due to their unique mechanism(s) of action and their capability to treat drug-resistant bacteria (Baindara et al., 2015; Fickers, 2013). Notably, AMPs display low toxicity towards mammalian cells (Grosset & Leventis, 1983; Yew & Chu Leung, 2006) and show potent bactericidal activity against pathogens as well as potential pathogens. There are about 200 bacteriocins reported with diverse amino acid sequences that are available at different bacteriocin databases, including www.bactibase.pbfa-lab-tun.org. Though AMPs are predominantly reported from the strains belonging to the genera Lactobacillus and Bacillus (Klaenhammer, 1993; Teixeira et al., 2013; Zhao et al., 2012), members of other genera such as Paenibacillus and Brevibacillus were also found to produce bacteriocins (Baindara et al., 2015; Singh et al., 2012). Accordingly, members of the genus Brevibacillus, specifically, strains of species B. laterosporus are known for their beneficial functions (Ruiu et al., 2014) and antimicrobial substance production. Moreover, it is known as a bio-control agent for nematodes and also used to treat fungal disease (Oliveira et al., 2004). Besides the production of antimicrobials such as bacteriocins, antibiotics and lipopeptide antibiotics, members of B. laterosporus also produce thrombin inhibitors and anti-tumour agents (Kamiyama et al., 1994;
Laterosporulolin10, a defensin like Class IId bacteriocin

Umezawa & Takeuchi, 1987). In fact, the whole-genome sequencing of this bacterial species reveals its potential to produce diverse antimicrobial peptides, polyketides and toxins (Shida et al., 1996; Smirnova et al., 1996; Van Belkum et al., 2011). Recently, we have characterized a class IId bacteriocin from a *B. laterosorus* strain GI-9 and named it laterosporulolin. This bacteriocin displayed structural homology and sequence similarity to various human defensins (Singh et al., 2014). The laterosporulolin produced by *B. laterosorus* strain GI-9 displayed a broad range of antibacterial activity through membrane permeabilization (Carrillo et al., 2003; Singh et al., 2014). However, it is important to test the ability of bacteriocins, including the lantibiotics, for their efficiency in inhibiting the growth of *M. tuberculosis* (Donaghy, 2010; Piper et al., 2012). Therefore, in the present study, we report the characterization and antimicrobial activity of laterosporulolin10 against various potentially pathogenic bacteria.

**METHODS**

**Bacterial strain and identification.** An antimicrobial substance producing strain SKDU10 was isolated from a rhizosphere soil sample. Phenotypic characteristics used for strain identification were determined as mentioned in Bergey's manual of systematic bacteriology. For genotypic identification, the 16 s rRNA gene was amplified and sequenced as described earlier (Sharma et al., 2012). The sequence (accession no. HF545882) was compared to other close relatives by phylogenetic analysis (Theodore et al., 2014). Indicator strains used in this study, including *Staphylococcus aureus* (MTCC 1430), *Bacillus subtilis* (MTCC 121), *Pseudomonas aeruginosa* (MTCC 1934), *Vibrio cholerae* (MTCC 3904), *Escherichia coli* (MTCC 1610), *Canidia albicans* (MTCC 1637), *Saccharomyces cerevisiae* (MTCC 170), *Fusarium oxysporum* (MTCC 2773) and *Aspergillus niger* (MTCC 281), were obtained from Microbiological Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. While all bacterial indicator strains were grown on nutrient agar (NA, Himedia, India) medium, fungal indicator strains were grown on potato dextrose agar (PDA, Himedia, India) at 30 °C under aerobic conditions. All strains were maintained as glycerol stocks at −70 °C.

**Antimicrobial peptide production and activity assay.** A growth curve was established up to 30 h to test the antimicrobial peptide production at different growth phases using nutrient broth medium (NB, Himedia, India). To test the effect of different carbon and nitrogen sources on antimicrobial production by strain SKDU10, a 0.5 % concentration of different substrates including glucose, lactose, yeast extract, peptone and beef extract was added to the minimal medium (composition (g/l): Na2HPO4·2H2O, 7.9; KH2PO4, 3.0; NaCl, 0.5; NH4Cl, 1.0; pH 7.2). Antimicrobial production ability of the strain was measured as the diameter of the inhibition zone by performing an antimicrobial bioassay using cell-free fermented broth (CFB). Strain SKDU10 was grown in minimal medium containing different carbon and nitrogen sources for 48 h, and the CFB obtained was tested by well diffusion assay. The CFB obtained by growing strain SKDU10 in NB (pH 7.0) was also used to test the activity against indicator strains, including *S. aureus* (MTCC 1430), *B. subtilis* (MTCC 121), *P. aeruginosa* (MTCC 1934), *V. cholerae* (MTCC 3904), *E. coli* (MTCC 1610), *C. albicans* (MTCC 1637), *S. cerevisiae* (MTCC 170), *F. oxysporum* (MTCC 2773) and *A. niger* (MTCC 281). Bacterial indicator strains were grown to obtain an OD600 of 0.2, and subsequently 50 µl of culture aliquots (final concentration, 104–105 CFU ml−1) were used for well diffusion assay. All NA plates for antimicrobial activity assay were incubated at 30 °C overnight under aerobic conditions.

**Purification of bacteriocin.** The bacteriocin was extracted from cell-free supernatant (CFS) using 2 % (w/v) of activated Diaion HP-20 as mentioned earlier (Singh et al., 2012). This crude extract was purified by gel filtration chromatography using a manually packed Sephadex G-50 (GE healthcare, USA) column. The peptide was eluted with 50 mM NaCl at a flow rate of 1.0 ml min−1 and fractions showing antimicrobial activity were collected, pooled and concentrated. Subsequently, the extract was dialysed using a Float-A-Lyzer G2 (MWCO 0.5–1 kD; Spectrum laboratories, USA) to remove excess salt. Desalted peptides were finally purified by HPLC (1260 Infinity, Agilent Technologies, USA) using a reverse-phase, semi-preparative C18 column (250 mm×10 mm×150 Å, Venusil, Agela technologies) as described earlier (Bairdara et al., 2015). The concentration of purified peptide was determined using a Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). Peptide quantified between 0–16 µM concentration was used for further studies, including determination of minimum inhibitory concentration (MIC), molecular mass and N-terminal sequencing as mentioned below. This peptide was also used for an in-gel activity assay against *S. aureus* MTCC 1430 as described above (Bairdara et al., 2013).

**Mass spectrometry analysis of bacteriocin.** Matrix-assisted laser desorption ionization (MALDI) mass spectrometry (AB SCIEX, 5800 TOF/TOF, USA) was used primarily to determine the molecular weight of AMP. The peptide was re-suspended in methanol, and 4 µl of this solution was mixed with equal amounts of matrix (CHCA, 10 mg ml−1). From this mixture solution, 1.0 µl was spotted onto a MALDI stainless steel sample plate and allowed to air-dry prior to MALDI analysis (Mandal et al., 2009). The spectra were recorded in the positive ion linear mode.

**N-terminal sequencing and analysis.** Upon separation on Tricine-SDS-PAGE (16 % with 6 M urea), the peptide was transferred to PVDF membrane (Bio-Rad, USA), rinsed with Milli-Q water and stained with amido black (Sigma, USA) for 2–3 min. The membrane was processed for N-terminal sequencing as mentioned earlier (Singh et al., 2012).

**Minimum inhibitory concentration (MIC) and killing kinetics.** The lowest concentration that inhibited 90 % of growth was considered as MIC and was determined using microtitre plate dilution assay. Protein concentration was estimated using the BCA kit (Thermo Scientific, USA) as described by the manufacturer and confirmed with the extension coefficient of the peptide sequence. Lysozyme protein standards (Thermo Scientific, USA) were used as references for estimation of protein concentration. Purified AMP was in the range 0–16 µM to determine the MIC values against various indicator strains. To determine the killing kinetics of bacteriocin, indicator strain *S. aureus* MTCC 1430 (~2×10^4 cells) was treated with bacteriocin at different concentrations, including 4, 8 and 20 µM, in a time-dependent manner (for 30, 60 and 90 min). Upon incubation, cells were pelleted by centrifugation (8000 g) and washed with PBS (Gibco, USA), subsequently serially diluted and plated at different dilutions on NA plates. Untreated cells were used as a positive control and were processed along with treated cells for CFU counts. The experiment was performed in triplicate and repeated as three individual sets, then analysed for final results (Singh et al., 2014).

**MIC determination against MtB using microplate Alamar blue assay.** MtB H37Ra, MtB H37Rv, and *M. smegmatis* MC2 155 (Msmeg) were cultured in Middlebrook 7H9 media (supplemented with 10 % OADC- along with 0.05 % Tween 80) for 48 and 16 h duration (for MtB and Msmeg strains, respectively) to obtain an OD600 of 0.2, and used to set up the microplate alamar blue assay (MABA) (Pettit et al., 2005). One hundred microlitres of culture was added to each well of a polypropylene 96-well plate (Eppendorf, USA) followed by serial dilution of the laterosporulolin10 from 10–0.0015 µg ml−1 to determine the
MIC. The plate was incubated for 24 h (for M. smegmatis M153 strain) and 72 h (for Mtb H37Ra and Mtb H37Rv strains) at 37 °C. After the incubation, 50 µl of 0.02 % re-sazatin was added to each well and the plate was monitored from 12 to 24 h for change in colour. Rifampicin was used as a positive control. The absorbance at 600 nm was measured using an ELISA reader (Thermo Scientific, USA) and the percentage of inhibition was calculated. The experiments were performed in triplicate and repeated at least three times.

**Determination of fractional inhibitory concentration (FIC).** As rifampicin is among the frontline drugs for Mtb H37Rv, further testing was performed to determine whether a combination of rifampicin and laterosporulin10 could reduce the MIC (MIC of rifampicin is 0.025 µM) values of rifampicin against Mtb H37Rv, MABA. Varying concentrations of rifampicin (0.003125–0.2 µM) and laterosporulin10 (0.031–4.0 µM) were used individually or in combination to check the additive effect of latero-sporulin10 and rifampicin. Next, to distinguish between the additive and synergistic effects of laterosporulin10 with rifampicin, we determined the \( \sum \text{FIC} \) values (Ghatuwedi et al., 2011). The \( \sum \text{FIC} \) values were calculated using the following formula: \( \sum \text{FIC} = \text{FIC}_B + \text{FIC}_A \). Wherein \( \text{FIC}_A \) equals the MIC of drug A in combination/MIC of drug A alone, and \( \text{FIC}_B \) equals the MIC of drug B in combination/MIC of drug B alone. Laterosporulin10 was considered as drug A and rifampicin as drug B. The interpretation of \( \sum \text{FIC} \) values was as follows: <0.5, synergistic; >0.5 to <4.0, indifferent (no antagonism); and ≥4.0, antagonistic.

**Sensitivity of AMP to temperature, pH and proteolytic enzymes.** The sensitivity of purified AMP to pH, temperature and proteolytic enzymes was confirmed by performing agar-well diffusion assay. To determine the pH stability, aliquots of purified peptide (1 mg ml\(^{-1}\)) were adjusted to pH 2.0–12.0 in increments of 2 pH units (using 10 mM HCl or NaOH) followed by incubation at room temperature for 4 h, and the leftover activity was measured upon neutralizing the sample to pH 7.0. For the thermal stability assay, aliquots of purified peptide (1 mg ml\(^{-1}\)) were exposed to 0.02 % SDS in PBS, diluted serially and plated on 7H11 agar plates. The interpretation of \( \sum \text{FIC} \) values was as follows: <0.5, synergistic; >0.5 to <4.0, indifferent (no antagonism); and ≥4.0, antagonistic.

**Survival of Mtb H37Rv strain in macrophages.** RAW 264.7 murine macrophages were seeded in 6-well plates (5×10\(^4\) cells) and infected with Mtb H37Rv strain (1:10 MOI) for three hours (Kumar et al., 2008). The cells were then treated with 100 µg ml\(^{-1}\) gentamycin for 45 min to remove extracellular bacteria. The cells were independently stimulated with either 0.025 and 0.125 µM of rifampicin or 0.5, 2.5 and 5 µM of laterosporulin10. After 48 h, the cells were lysed using 0.06 % SDS in PBS, diluted serially and plated on TH11 agar plates.

**Mammalian cell toxicity.** Cultures of RAW 264.7 murine macrophage were subjected to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen, life technologies, USA) assay to determine the number of surviving cells after treatment with laterosporulin10. The cells were plated in 96-well BD Falcon vessels (5×10\(^3\) cells/well) using DMEM medium (Gibco, USA) supplemented with 10 % fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin cocktail (Sigma, USA). Cultures were incubated under standard conditions (37 °C and 5 % CO\(_2\)). After 24 h, the growth medium was replaced with fresh medium containing various concentrations of laterosporulin10 (1–40 µM). The medium without peptide was used as a negative control and 1 % Triton X–100 was used as positive control. The plates were incubated for an additional 24 h, and then to each well 20 µl of MTT solution (5 mg ml\(^{-1}\) in PBS) was added, and the plates were incubated for 3 h at 37 °C. Subsequently, the MTT containing medium was removed and 50 µl of DMSO were added to each well. To assess the percentage of live cells in the samples, the absorbance (590 nm) was assessed as described earlier (Baindara et al., 2015).

**Haemolysis assay.** Rabbit (New Zealand white) blood samples were collected in test tubes containing EDTA and processed as mentioned earlier (Singh et al., 2014). The positive control used was 1 % Triton X–100 (G Biosciences USA) in water and sterile PBS as a negative control. After incubation at 37 °C, all treated samples were centrifuged at 1500 rpm for 5 min and supernatants were transferred to a fresh 96-well plate to examine erythrocyte lysis by spectrophotometer (Thermo Fisher Scientific, USA) at a wavelength of 541 nm (Smolarczyk et al., 2010).

**ATP determination assay.** Actively growing cultures (1 ml) of S. aureus MTCC 1430 (16 h culture) and Mtb H37Rv strain (0.2 OD\(_{600}\) 48 h grown culture) were treated with different concentrations of laterosporulin10 (0.5, 2.5 µM for Mtb H37Rv and 4, 8, 20 µM for S. aureus MTCC 1430) in a time-dependent manner (10, 60 min for Mtb H37Rv and 30, 60, 90 and 120 min for S. aureus). After incubation, the cells were separated by centrifugation (8000 g) and washed with PBS (Gibco, USA) twice. The cells recovered from the pellets were resuspended in 50 µl of lysis buffer (Promega, USA), mixed gently for 1 min and centrifuged (8000 g). The supernatant was collected and 10 µl of the dilution was added to standard reaction solution (90 µl) from the ATP determination kit (Invitrogen, Molecular Probes, USA). Oxyfluorcin concentration was determined after measuring luminescence on a white plate using a Lumat LB 9501 luminometer (Promega, USA). The protein content was assayed using the BCA kit (Thermo Scientific, USA) and sample luminescence was expressed as RLU/mg of protein. Data from three separate measurements were used to calculate average values.

**NAD/NADH, NADP/NADPH assay.** One millilitre of 0.2 OD\(_{600}\) cultures of Mtb H37Rv strain was treated with 0.5 µM of laterosporulin10 in a time-dependent manner (10 and 60 min). After incubation, cells were pelleted down at 10 000 rpm for 5 min and the pellet was resuspended in 1 ml lysis solution (0.2 N NaOH and 1 % dodecyl trimethyl ammonium bromide, DTAB) followed by cell lysis using an OMNI bead ruptor (5 cycles, 45 s each). The lysate was filtered and removed from BSL3. The protein was estimated using the BCA method (kit procured from Sigma, USA). Once the protein was estimated, the lysate was used for estimation of NAD/NADH (NAD/NADH-Glo™ assay kit, Promega, USA), NADP/NADPH (NADP/NADPH-Glo™ assay kit, Promega) as per the manufacturer’s protocol.

**Preparation of cells for SEM.** Since a high cell density is needed to observe EM images, Mtb H37Rv cultures of mid-exponential growth phase (about 10\(^9\) to 10\(^10\) CFU ml\(^{-1}\)) were centrifuged at 8000 rpm for 10 min, and the cells were washed twice with PBS. Finally, the cell pellet was diluted with PBS to obtain a cell density of 10\(^6\) CFU ml\(^{-1}\). Subsequently, the cells were treated with different concentrations (0.5 and 1.0 µM) of laterosporulin10 in a time-dependent manner (10 and 30 min) and incubated at 37 °C (Hartmann et al., 2010). Untreated controls were prepared by diluting the cell pellet in PBS at a cell density of 10\(^8\) CFU ml\(^{-1}\). Mtb H37Rv cells were immobilized on polylysine (0.1 % wt vol\(^{-1}\) aqueous solution, Sigma, USA)-coated cover slips and washed thrice with ice-cold PBS, then fixed in modified Karnovsky fixative (4 % paraformaldehyde, 1 % glutaraldehyde and 0.2 M sodium cacodylate buffer, pH 7.4) for 2 h and dehydrated in graded ethanol (30–100 %). Ethanol-dehydrated samples were freeze-dried and processed further by scanning electron microscopy utilizing tertiary butyl alcohol as an intermediate fluid. Cover slips were placed on aluminium stubs using silver paint and sputter coated with gold. The cells were then observed and...
photographed using an S-260, Leica Cambridge scanning electron microscope (Raje et al., 2006).

**Preparation of cells for TEM.** Mid-exponential growth cultures of *S. aureus* MTCC 1430 and *Mtb* H37Rv strain were centrifuged at 8000 g for 10 min, and cells were collected and washed twice with PBS (Gibco, USA). The cell pellet obtained after final washing was diluted with PBS to obtain a cell density of approximately $10^8$ CFU ml$^{-1}$ and treated with different concentrations of laterosporulin10 (0.5, 1.0 µM for *Mtb* H37Rv and 4, 8 µM for *S. aureus* MTCC 1430) in a time-dependent manner (10 and 30 min) at 37°C. After treatment, the cell pellet was washed with PBS and fixed in modified Karnovsky’s fixative. The cells were further fixed in 1 % osmium tetroxide (Sigma-Aldrich, USA) in 100 mM PBS and embedded in 2 % agarose. After making blocks with ACM I and ACM II (Sigma-Aldrich, USA), sectioning (70 nm) was performed using an Ultramicrotome (Leica EM UC7, USA). Subsequently, cells were stained with 0.1 % (w/v) PTA (sodium phosphate tungstate, Sigma) on a carbon-coated copper grid (300 mess, Polysciences, USA) and observed under a JEOL JEM 2100, 200 kV transmission electron microscope (TEM) at a resolution of 0.2–0.5 µM (Raje et al., 2006). Untreated cells were used as control.

**FACS analysis by flow cytometry.** Cell membrane integrity was determined using propidium iodide (Invitrogen, USA). Propidium iodide (PI) is a nucleic acid stain that binds to DNA by intercalating between the bases and is membrane impermeant that is excluded from viable cells (Arndt-Jovin & Jovin, 1989). *S. aureus* MTCC 1430 cells of mid-exponential growth phase were centrifuged at 8000 rpm for 10 min and the cell pellet was washed three times with PBS (GIBCO's PBS without Ca$^{2+}$ and Mg$^{2+}$ ions). The cell pellet was diluted with PBS to obtain a cell density of $10^6$ CFU ml$^{-1}$ and subsequently treated with 4 µM of laterosporulin10 in a time-dependent manner (5, 10 and 30 min) and incubated at 37°C. Upon incubation at the above-mentioned time intervals, the treated cells were pelleted down, washed with PBS three times and loaded with 5 µl of PI (1 mg ml$^{-1}$). After 15 min of incubation with PI, the cells were centrifuged at 8000 rpm for 10 min and washed with PBS three times. Untreated cells in PBS were used as control. Subsequently, the treated cell pellet was diluted with PBS and analysed immediately using a flow cytometer (BD Acuri, USA).

**PEGylation.** To determine the amount of free cysteine in AMP, the maleimide PEG (MalPEG, 5 kDa) (Sigma-aldrich, USA) molecule was reacted with different states (native, reduced, unfolded and reduced 2000 mAU (a)) 1500 1000 500 0 (b) Molecular weight determination on a Shodex 802 HPLC column. Laterosporulin10, elution profile suggested it as a monomer. (c) Tricine-SDS-PAGE and in-gel activity assay of the purified laterosporulin10. Molecular weight marker along with laterosporulin10 (L1), portion of the SDS-PAGE gel overlaid with 0.8 % soft agar containing *S. aureus* MTCC 1430, (L2) showing zone of clearance. All electrophoresis and activity assays done with purified peptide dissolved in PBS. (d) MALDI-TOF analysis of the purified peptide from *B. laterosporus* strain SKDU10 shows mass (m/z) of 6.0 kDa.

**Fig. 1.** Purification, molecular weight determination and in-gel activity assay of laterosporulin10. (a) Reverse-phase HPLC profile of laterosporulin10 (inset shows the antimicrobial activity against *S. aureus* MTCC 1430). (b) Molecular weight determination on a Shodex 802 HPLC column. Laterosporulin10, elution profile suggested it as a monomer. (c) Tricine-SDS-PAGE and in-gel activity assay of the purified laterosporulin10. Molecular weight marker along with laterosporulin10 (L1), portion of the SDS-PAGE gel overlaid with 0.8 % soft agar containing *S. aureus* MTCC 1430, (L2) showing zone of clearance. All electrophoresis and activity assays done with purified peptide dissolved in PBS. (d) MALDI-TOF analysis of the purified peptide from *B. laterosporus* strain SKDU10 shows mass (m/z) of 6.0 kDa.
Whole-genome sequencing, assembly and annotation. The genomic DNA was isolated using DNA extraction kits (Zymo Research, USA), and quality was assessed using agarose gel electrophoresis, Qubit and Nanodrop. The input of 1 μg of genomic DNA from each sample was used. Standard protocol for the Nextera XT DNA sample preparation kit was used for library construction. Purified fragmented DNA was used as a template for a limited-cycle PCR using Nextera primers and index adaptors. Cluster generation and sequencing of libraries were performed on the Illumina MiSeq platform (Illumina, San Diego, USA) at 40 °C for 1 h was used for unfolding of native peptide (Yoneyama et al., 2009).

To extract the putative bacteriocin encoding ORF from the draft genome sequence, a homology search was carried out using the Tblastn function against the draft genome sequence of SKDU10 as mentioned earlier (Singh et al., 2012). ClustalW was used to carry out sequence alignment of nucleotide and amino acid sequences.

Statistical analysis. All comparisons were based on mean ± standard deviation of the mean (sd). Parametric data were analysed using one-way analysis of variance (ANOVA) with Bonferroni post-test method for comparison between groups. Column statistics for non-parametric data were analysed by D’Agostino & Pearson omnibus normality test along with one sample t-test. Results were considered significant at P<0.05 in all experiments. All experiments were done independently in triplicate.

RESULTS

Characterization of bacterial strain SKDU10

In an effort to look for the antimicrobial-producing strains from complex environments, we isolated a few strains with inhibitory zones in their peripheral colony. One of the bacterial strains designated as SKDU10 was studied in detail.
for its ability to inhibit the growth of other bacterial indicator strains. Before proceeding with characterization of antimicrobial substance, the morphological characterization of strain SKDU10 revealed it as a Gram-positive, rod-shaped, endospore-forming bacterium with the ability to grow under aerobic and anaerobic conditions. Both 16S rRNA and rpoB gene sequences showed high identity with the strain of B. laterosporus DSM 25. However, it displayed less than 97% similarity with other species of the genus Brevibacillus. Strain SKDU10 formed a distinct cluster with B. laterosporus DSM 25 in a neighbour-joining phylogenetic tree constructed using 16S rRNA (Fig. S1, available in the online Supplementary Material) and rpoB gene sequences. Strain SKDU10 displayed differences with B. laterosporus DSM 25 in phenotypic properties, such as growth at pH 5 and 4% NaCl, casein hydrolysis and utilization of sugars like mannose. Antimicrobial substance production by strain SKDU10 in NB was initiated towards the late-logarithmic phase (Fig. S2). Similar observations were also found with antimicrobial substance grown on minimal medium with different substrates.

**Purification and characterization of antimicrobial substance**

The antimicrobial substance was recovered with hydrophobic interaction chromatography using activatedDiaion HP-20 beads as crude extract, which was purified subsequently by size exclusion chromatography and finally using semi-preparative RP-HPLC (Fig. 1a). While absorption wavelength and HPLC elution profile indicated the antimicrobial substance to be a peptide, the gel filtration elution profile showed a single peak with low molecular weight (Fig. 1b). Accordingly, a single band observed in gel electrophoresis displayed antimicrobial activity during the in-gel activity assay using S. aureus (MTCC 1430) as a reference strain (Fig. 1d). The mass of peptide was confirmed as 6061.37 Da by MALDI analysis (Fig. 1c), and differed from laterosporulin, a class IId defensin-like bacteriocin produced by another strain of B. laterosporus designated GI-9, and therefore it was named laterosporulin10. Antimicrobial activity of the purified laterosporulin10 was not affected following exposure to 121°C for 15 min (Fig. S3a). Similarly, it was found to be stable between pH 2.0 and 12.0 (Fig. S3b). Studies on tolerance of the peptide to proteolytic enzymes did not show any decrease in inhibition activity of laterosporulin10 upon incubation for 6 h with trypsin and proteinase K (Fig. S3c, d).

**Minimum inhibitory concentration and killing kinetics**

The quantified laterosporulin10 was used to determine the minimum inhibitory concentrations required for various indicator strains. It displayed effective inhibition activity towards Gram-positive indicator strains as the LD50 values showed 4 to 6 µM concentrations against S. aureus (MTCC 1430) and B. subtilis (MTCC 121), respectively, but the growth of Gram-negative indicator strains was not affected even at a concentration of 16 µM (Fig. 2a). Furthermore, the MIC for E. coli was found to be ~100 µM. However, the bactericidal kinetic studies obtained for laterosporulin10 revealed its effectiveness against cells of S. aureus as more than 90% of bacterial load reduced within 30 min of treatment at 4 µM concentration of the bacteriocin (Fig. 2b). There was no reduction in CFU count observed for negative control incubated without any bacteriocin. Maximum cell death was observed within 30 min of treatment for different concentrations of laterosporulin10.
Laterosporulin10 found to be active against Mtb H37Rv strain

The MIC of laterosporulin10 against the Mtb H37Rv strain was measured using MABA, which utilizes a redox potential indicator dye resazurin that fluoresces and changes colour upon reduction (blue to pink) due to cell growth. Therefore, while change of colour from blue to pink indicates cell growth, the blue colour shows bacterial killing or inhibition of bacterial growth. We observed that laterosporulin10 was highly efficient in killing Mtb H37Rv strain with a LD50 of 0.5 µM (Fig. 4). Since this LD50 was eightfold lower than the LD50 obtained for S. aureus MTCC 1430 (4 µM), we investigated whether laterosporulin10 kills the M. smegmatis MC2 155 strain, a saprophytic mycobacterial species, with similar efficiency. To our surprise, laterosporulin10 revealed an MIC of 45 µM against M. smegmatis MC2 155 (Fig. S4). Thus the MIC for M. smegmatis MC2 155 was about 50-fold higher than that observed for the Mtb H37Rv strain. These findings are very interesting as laterosporulin10 has species-specific inhibitory activity against the Mtb H37Rv strain. Killing of Mtb H37Rv strain was also confirmed through plating and determination of CFU in the presence or absence of laterosporulin10. Since rifampicin is one of the frontline drugs used for Mtb H37Rv, to determine whether a combination of laterosporulin10 and rifampicin could reduce the MIC of rifampicin (MIC of rifampicin is 0.025 µM) against Mtb H37Rv strain, MABA was performed, wherein varying concentrations of rifampicin

Laterosporulin10 disrupts ATP homeostasis in S. aureus MTCC 1430

As the membrane is the target for most bacteriocins, we tested whether laterosporulin10 acts by disrupting the cell membrane of sensitive strains like S. aureus MTCC 1430. Since membrane disintegration often results in depletion of intracellular ATP, we determined the change in ATP content following treatment of S. aureus MTCC 1430 cells with laterosporulin10. The log phase cultures of S. aureus MTCC 1430 were treated with laterosporulin10 at 4, 8 and 20 µM for 30, 60, 90 and 120 min and subsequently their ATP content was determined. We found that treatment of S. aureus MTCC 1430 cells with 4 and 8 µM of laterosporulin10 led to the disruption of ATP homeostasis within 90 min, whereas treatment with 20 µM resulted in disruption of ATP homeostasis as early as 30 min (Fig. 2c). Accordingly, reduction in ATP levels supports a membrane-associated mechanism for laterosporulin10 in regard to bacterial killing. Furthermore, loss of membrane integrity induced by laterosporulin10 was also evaluated using nucleic acid stain PI and fluorescence was detected after treatment at 10, 30 and 60 min. The group without bacteriocin was used as a negative control, where only 0.3 % cells showed PI uptake. Results confirmed that the membrane was significantly compromised in the presence of laterosporulin10, as intake of PI was observed for 39.9 % of the total cell population within 30 min of treatment (Fig. 3).
bars. P samples were prepared in PBS. Error bars represent while Mtb H37Rv treated with 0.025 and 0.125 Mtb H37Rv cells without treatment served as negative control electron microscopy experiments, we determined the ability cion, and less than 20 % haemolysis was observed with 100 was not observed at up to 20 µM concentration of bacterio- of control S. aureus MTCC 1430 cells showed a smooth and intact surface, cells treated with 4 µM laterosporulin10 for durations of 10 and 30 min displayed significant alterations in cell morphology, including formation of cell clumps along with debris material (Fig. 8a). The results of SEM analysis of the Mtb H37Rv strain treated with 0.5 µM laterosporulin10 for durations of 10 and 30 min also showed cell clumping with cell debris material (Fig. 8b). In accordance with this, TEM micrographs revealed disintegration of cell membrane and subsequent total cell lysis of the Mtb H37Rv strain (Fig. 8c), suggesting that laterosporulin10 acts against S. aureus MTCC 1430 and Mtb H37Rv strain by disrup- tion of their cell membranes.

Laterosporulin10 is a non-haemolytic and non-cytotoxic bacteriocin

Since laterosporulin10 demonstrated anti-mycobacterial activity against the intracellular Mtb H37Rv strain, it is important to investigate its effect on mammalian cells. Towards this, we investigated whether laterosporulin10 has any haemolytic activity. Fresh blood drawn from New Zealand white rabbits was incubated with different concentrations of laterosporulin10 (1–100 µM) for 24 h. Haemolysis was not observed at up to 20 µM concentration of bacteriocin, and less than 20 % haemolysis was observed with 100 µM of laterosporulin10 (Fig. 3d). Since the concentration used was several fold higher compared with the MIC against Mtb H37Rv strain and S. aureus MTCC 1430, this bacteriocin could potentially be used in animals for further study of its antimycobacterial potential. In an attempt to test the toxicity of laterosporulin10 against nucleated cells, we used of laterosporulin10 to disrupt ATP, NAD/NADH and NADP/NADPH homoeostasis in the Mtb H37Rv strain. Towards this, log phase cultures of Mtb H37Rv strain were treated with laterosporulin10 for 10, 30 and 60 min with 0.5 and 2.5 µM, and the lysates were measured to determine the ATP level and NAD/NADH. Results showed that in contrast to S. aureus MTCC 1430, that required 90 min for the disruption of ATP homeostasis upon treatment with 4 or 8 µM, laterosporulin10 efficiently disrupted Mtb H37Rv ATP homeostasis in less than 10 min at lower concentra- tions (Fig. 7a). These findings were further supported by the observation that the NAD/NADH and NADP/ NADPH ratios were also disrupted upon treatment with laterosporulin10 (Fig. 7b and c).

Laterosporulin10 causes alterations in bacterial cell membrane

We observed that laterosporulin10 is capable of inhibiting bacterial growth within one hour. Therefore, to capture interactions of laterosporulin10 and cell membranes, we performed scanning and transmission electron microscopy (SEM and TEM, respectively). Since laterosporulin10 showed activity primarily against Gram-positive bacteria, cells of S. aureus MTCC 1430 treated with laterosporulin10 were observed by electron microscopy. While TEM images of control S. aureus MTCC 1430 cells showed a smooth and intact surface, cells treated with 4 µM laterosporulin10 for durations of 10 and 30 min displayed significant alterations in cell morphology, including formation of cell clumps along with debris material (Fig. 8a). The results of SEM analysis of the Mtb H37Rv strain treated with 0.5 µM laterosporulin10 for durations of 10 and 30 min also showed cell clumping with cell debris material (Fig. 8b). In accordance with this, TEM micrographs revealed disintegration of cell membrane and subsequent total cell lysis of the Mtb H37Rv strain (Fig. 8c), suggesting that laterosporulin10 acts against S. aureus MTCC 1430 and Mtb H37Rv strain by disrup-

Laterosporulin10 disrupts ATP, NAD/NADH and NADP/NADPH homeostasis in Mtb H37Rv strain

Since laterosporulin10 kills the Mtb H37Rv strain very efficiently by disrupting the cell membrane, as shown by the electron microscopy experiments, we determined the ability

![Image](image-url)

**Fig. 6.** Effect of laterosporulin10 on survival of intracellular Mtb H37Rv. Bars represent the log CFU ml⁻¹ after treatment with 0.025, 0.125 µM of rifampicin and 0.5, 2.5 µM of laterosporulin10. Mtb H37Rv cells without treatment served as negative control while Mtb H37Rv treated with 0.025 and 0.125 µM, MIC values of rifampicin served as positive control. Bacterial cells and peptide samples were prepared in PBS. Error bars represent SD, *, P<0.05 in comparison to the negative control and indicated above bars. (0.003125–0.2 µM) and laterosporulin10 (0.31–4.0 µM) were used alone or in combination. Interestingly, the treatment in combination led to a fourfold decrease (0.00625 µM) in the MIC of rifampicin in the presence of 0.25 µM of laterosporulin10 (Fig. 5). To distinguish between the additive and synergistic effects of laterosporulin10 and rifampi- cin, we determined FIC values. Strikingly, the effect of laterosporulin10 and rifampicin was revealed as being synergistic (∑FIC=0.275). Furthermore, we investigated whether laterosporulin10 is capable of killing the Mtb H37Rv strain in macrophages. Here, we infected RAW 264.7 murine macrophages with Mtb H37Rv at a MOI of 1:10 and then treated them with 0.5 and 2.5 µM of laterosporulin10 and rifampicin independently for 48 h. Upon incubation, the macrophage cells were lysed, and the lysates were plated on 7H11 agar for monitoring the CFU of the Mtb H37Rv strain. We observed that laterosporulin10 killed the intracellular Mtb H37Rv strain at both concentrations, though the killing was significantly higher at 2.5 µM (Fig. 6). These experiments suggest that laterosporulin10 can enter phagosomes and efficiently kill the intracellular Mtb H37Rv strain.

http://mic.microbiologyresearch.org 1293
RAW 264.7 (murine macrophage). Remarkably, laterosporulin10 did not show any cytotoxic effect up to 30 µM (Fig. S5), which is a significantly higher concentration than the MIC<sub>50</sub> observed for different bacterial strains tested, including Mtb H37Rv. More than 80 % cell viability was observed in three independent experiments.

**Genetic characterization of putative laterosporulin10 biosynthetic gene cluster**

In order to identify the amino acid composition of laterosporulin10 and its identity with other bacteriocins, we performed N-terminal amino acid sequencing and obtained a partial sequence composed of ACVNZCP-DAIDR. The 12 amino acid N-terminal sequence displayed identity with only laterosporulin. However, this partial sequence was used to identify the biosynthetic gene encoding the bacteriocin from the draft genome sequence of strain SKDU10 along with other genes involved in biosynthesis (Fig. 9a). The identified ORF contained 165 nucleotides with a conserved Shine–Dalgarno sequence, 8 bp upstream of this ORF encoding (Fig. 9b). Computational translation of this ORF resulted in a protein of 54 amino acids with conserved motif PDAI, and six cysteine amino acids at conserved positions with the probability of their involvement in disulphide bond formation as observed for laterosporulin. However, the peptide composition differed with the amino acid composition of laterosporulin. Though the biosynthetic cluster of this novel bacteriocin contained identical transcriptional regulators, the ABC transporter and dehydrogenase gene as observed in laterosporulin, it contained a higher number of cationic amino acids and displayed low similarity (57.6 %) with laterosporulin, representing a

---

**Fig. 7.** Determination of dinucleotide homeostasis disruption ability of laterosporulin10 in Mtb H37Rv. (a) ATP release assay. Bars represent amount of ATP released after treatment with 0.5 and 2.5 µM laterosporulin10 (10 and 60 min for both). (b) NAD/NADH assay. Bars represent ratio of NAD/NADH after treatment with 0.5 µM of laterosporulin10 (10 and 60 min). (c) Bars represent ratio of NADP/NADPH after treatment with 0.5 µM of laterosporulin10 (10 and 60 min). Untreated cells used as control. Bacterial cells and peptide samples were prepared in PBS. Error bars show sd. *, P<0.05 in comparison to the untreated controls, indicated above bars.
novel class IId bacteriocin. Overall, comparative studies suggested that laterosporulin10 shares homology with the antimicrobial peptides belonging to the beta-defensin family of mammals as observed in laterosporulin.

All cysteines are paired in laterosporulin10

Laterosporulin10 contained six cysteines with conserved position as observed for laterosporulin (Fig. 9a), and the mass obtained was 6 Da less than the theoretical mass of the peptide sequence deduced from the biosynthetic gene. The mass difference observed was attributed to the formation of three disulfide bonds as found in laterosporulin. The involvement of six cysteine molecules in the disulfide bond formation was confirmed by a PEGylation experiment, where the electrophoretic mobility of native and reduced PEGylated laterosporulin10 molecules showed the addition of PEG molecules under structured and unstructured conditions (Fig. S6a). In addition, increase in the molecular mass of laterosporulin10 under reduced conditions (Fig. S6b) also confirmed the intramolecular disulfide bond formation in laterosporulin10.

DISCUSSION

The emergence of resistance against predominant antimicrobials carries a significant threat to the control of communicable diseases. These observations are more relevant in the treatment of TB, that requires administration of multiple drugs for prolonged duration and where multidrug resistance is commonly observed. In the light of emerging drug resistance, genome sequencing has revolutionized our approach to the problem of understanding multidrug resistance in pathogens and the discovery of novel therapeutic agents. Therefore, the approach for identification of novel bacteriocin-producing genes is a powerful approach in identifying novel therapeutic agents. Both broad- and narrow-spectrum bacteriocins are often produced by bacterial strains to compete with other microbes, including different strains of the same species. In the recent past, antimicrobial peptides (Singh et al., 2012; Zhao et al., 2012) and lipopeptides (Desjardine et al., 2007) have been isolated and characterized from strains of B. laterosporus with broad-spectrum antimicrobial activity. In the present study, a bacteriocin-producing isolate was characterized to understand its ability to produce bacteriocin that inhibited the growth of S. aureus and other Gram-positive bacteria. However, among the various conventional indicator strains, laterosporulin10 effectively inhibited S. aureus (Fig. 2a), a bacterial strain considered to be a surrogate bacterium for the prediction of antimicrobial peptide activity against the Mtb H37Rv strain (Ramón-García et al., 2013). Therefore, we selected laterosporulin10 to test antimicrobial activity against the pathogenic M. tuberculosis H37Rv strain.

Though amino acid sequence analysis of the purified bacteriocin showed similarity with the laterosporulin (Singh et al., 2012), it significantly differed in both antimicrobial spectrum and amino acid composition. As observed for acidocinA, a bacteriocin isolated from Lactobacillus salivarius (Stern et al., 2006) that displayed activity against only Gram-positive bacteria, the natural replacement of amino acids in laterosporulin10 was also found to be less efficient against Gram-negative bacteria in comparison to laterosporulin. Growth inhibitory activity assays showed that laterosporulin10 exhibited strong anti-mycobacterial activity. The amino acid composition analysis of laterosporulin10 showed the predominance of hydrophobic amino acids, indicating the significance of the hydrophobic property in establishing their interaction with the bacterial cytoplasmic membrane. It is known in the literature that the Arg-rich cationic antimicrobial peptides exhibit activity towards both Gram-positive and Gram-negative bacteria (Nakatsuji & Gallo, 2014). Bioinformatics analysis suggested that the C-terminal was the active region of bacteriocin for antimicrobial activity, and therefore we speculate that the change in the C-terminal amino acid sequence is the primary reason for weak or no activity of laterosporulin10 against Gram-negative indicator strains. Here, it is important to note that replacement of serine (a polar amino acid) residue with neutral proline residue in laterosporulin10 might be another reason for weak or no activity of this bacteriocin towards Gram-negative strains. Interestingly, in comparison to other anti-mycobacterial drugs such as rifampicin and pyrazinamide, this bacteriocin is more specific in targeting the Mtb H37Rv strain. Thus, it fulfils the need for identification of species-specific bacteriocins, which is an important step in the direction of generating M. tuberculosis specific drugs. Earlier, NisinA was bio-engineered to create variants with species-specific activity against M. tuberculosis (Carroll et al., 2010a). Among other bacteriocins studied, lactacin 3147, produced by Lactococcus lactis subsp. lactis DPC3147 was found to have higher bactericidal activity against Mtb H37Rv compared to Mycobacterium avium subsp. paratuberculosis 09 890 and Mycobacterium kansasi (Carroll et al., 2010b). However, such identification is only a first step towards the creation of an efficient antimycobacterial drug and was never compared to combinatorial drug therapy. Rifampicin is one of the frontline drugs for M. tuberculosis, but its long-term use and dosage are related to interaction with antiretroviral drugs (Semvua et al., 2015), causing hepatotoxicity (Yew & Leung, 2006) and many other adverse effects (Grosset & Leventis, 1983). Many of these adverse effects can be controlled through the use of lower concentrations of rifampicin (Fresard et al., 2011) and, for these reasons, drugs that synergize with rifampicin should be included in the treatment regimen. However, it has been demonstrated that different bacteriocins isolated from B. circulans, P. polymyxa, L. salivarius, S. cirectus and E. faecalis possess antimycobacterial activity but that they are not able to inhibit mycobacterial growth inside macrophage cells (Sosnov et al., 2007). In the present study, we have shown that laterosporulin10 is capable of killing Mtb H37Rv strain residing in the phagosomes of murine macrophages. Nevertheless, the mechanism(s) utilized by laterosporulin10 to penetrate the cell membrane to target intracellular Mtb H37Rv remains unknown and is beyond the scope of this manuscript. As ATP
balance in bacterial cells is maintained in the mesosomes, reduction in ATP content is attributed to membrane disintegration (Kaplan et al., 2011; Lee et al., 2015). In fact, laterosporulin10 is non-toxic to macrophage cells at higher concentrations unlike other bacteriocins (Sosunov et al., 2007). The underlying mechanism, which ensures that laterosporulin10 targets only bacterial cells and not mammalian cells, is yet to be understood. A distinguishing component of this study is the identification of the genetic locus responsible for the biosynthesis of laterosporulin10. These findings will further help in identifying more natural variants, in the creation of recombinant variants of laterosporulin10 for improving its efficacy against Mtb, as was done earlier for Nisin A (Carroll et al., 2010a).

In agreement that most bacteriocins target the cell wall components (Cotter et al., 2013; Desjardine et al., 2007; Nilsen et al., 2003), disrupt membranes or alter membrane permeability (Ma et al., 2015; Maftah et al., 1993; Van Belkum et al., 1991), microscopic studies have revealed that laterosporulin10 also acts on the cell membrane of S. aureus and the Mtb H37Rv strain (Fig. 8) by disrupting cellular metabolic homeostasis. This activity is variable, based on the amino acid composition. Laterosporulin10 altered the membrane of the Mtb H37Rv strain, which is covered with a thick lipid layer having properties of a bipolar lipid membrane. The disruption of the Mtb H37Rv strain membrane was also demonstrated by alterations in the ATP levels and the NAD/ NADH and NADP/NADPH ratios that preceded the death of Mtb H37Rv cells. While a few studies have shown direct evidence of the interaction or involvement of AMP with different cell wall components (Martinez et al., 2008), other studies have speculated on the activity of bacteriocins or their interaction with cell wall/cell membrane components (Martinez et al., 2008; Yoneyama et al., 2011). On the other hand, haemolysis assays suggest that laterosporulin10 has no

Fig. 8. Determination of bactericidal effect of laterosporulin10 on S. aureus MTCC 1430 and the Mtb-H37Rv strain upon 30 min incubation (a) Transmission electron microscopy of S. aureus without laterosporulin10 treatment (i), with 4 µM (ii) and 8 µM (iii) laterosporulin10. (b) Scanning electron microscopy of Mtb H37Rv without laterosporulin10 treatment (i), with 0.5 µM (ii) and 1.0 µM (iii) laterosporulin10. (c) Transmission electron microscopy of Mtb H37Rv (sections) without laterosporulin10 treatment (i), with 0.5 µM (ii) and 1.0 µM (iii) laterosporulin10. Bacterial cells and peptide samples were prepared in PBS.
effect on RBCs as no haemolysis was observed, even at tenfold higher concentrations, on the Gram-positive strains or about 100-fold molar excess of the LD_{50} value on the Mtb H37Rv strain. Most importantly, this bacteriocin is not toxic to mammalian cells and kills the intracellular Mtb H37Rv strain, suggesting the potential of such compounds to treat drug-resistant tuberculosis. These new forms of peptides with improved antimicrobial activity and low cytotoxicity towards eukaryotic cells could be developed for use as therapeutic agents or in food preservation.

ACKNOWLEDGEMENTS

Financial assistance from the Department of Biotechnology (grant no. DBT/In-Bz/2013-16/16/SO-R1) and the Council of Scientific and Industrial Research (CSIR)-Network project (BSC-119) is duly acknowledged. We would like to thank Dr Vishakha Grover (Dr H. S. Judge Institute of Dental Sciences and Hospital, Punjab University, India) and Dr Pradip Kumar Singh (University of Maryland, USA) for useful discussions. We would also like to thank Mrs Sharanjeet Kaur and Dr Santi Mandal (Vidyasagar University, West Bengal, India) for their help in MALDI analysis of the peptide; Mrs Parmajeet Kaur for her help in N-terminal sequencing of the peptide; and Mr Randeep and Mr Anil Theophilus for their help in electron microscopy.

REFERENCES


Edited by: S. Gordon