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Title: Lactolisterin BU, a novel Class II broad spectrum bacteriocin from *Lactococcus lactis* subsp. *lactis* bv. diacetylactis BGBU1-4

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Runninig title: Aureocin like bacteriocin in lactococci

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ABSTRACT

Lactococcus lactis subsp. *lactis* bv. diacetylactis BGBU1-4 produces a novel bacteriocin, lactolisterin BU, with strong antimicrobial activity against many species of Gram-positive bacteria, including important food spoilage and food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus* sp. and streptococci. Lactolisterin BU was extracted from the cell surface of BGBU1-4 by propan-2-ol and purified to homogeneity by C18 solid phase extraction and reversed phase HPLC. The molecular mass of the purified lactolisterin BU was 5160.94 Da and an internal fragment, AVSWAWQH, as determined by N-terminal sequencing, showed low level similarity with existing antimicrobial peptides. Curing and transformation experiments revealed the presence of a corresponding bacteriocin operon on the smallest plasmid pBU6 (6.2 kb) of strain BGBU1-4. Analysis of the bacteriocin operon revealed a leaderless bacteriocin of 43 amino acids that exhibited similarity to bacteriocin BHT-B (63%), from *Streptococcus ratti*, a bacteriocin with analogy to aureocin A.

IMPORTANCE

Lactolisterin BU, broad spectrum leaderless bacteriocin produced by *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 strain, expresses strong antimicrobial activity against food spoilage and food-borne pathogens such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus* sp. and streptococci. Lactolisterin BU showed highest similarity with aureocin like bacteriocins produced by different bacteria. Operon for synthesis is plasmid located on the smallest plasmid pBU6 (6.2 kb) of strain BGBU1-4, indicating possible horizontal transfer among producers.

Keywords: bacteriocin, lactolisterin BU, antilisterial activity

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INTRODUCTION

Bacteria have the ability to produce an extraordinary array of different antagonistic compounds. These include bacteriocins, described as ribosomally synthesized hydrophobic peptides (1, 2) usually active against bacteria closely related to the producer. In addition, some bacteriocins have broader inhibitory spectra against medically important pathogens and food-spoilage bacteria (3). Based on structure, mechanism of action, biochemical and genetic characteristics, bacteriocins from lactic acid bacteria (LAB) are generally classified into two different groups: Class I bacteriocins (lantibiotics) contain unusual amino acids such as lanthionine and dehydrated amino acids as a result of post-translational modifications and Class II bacteriocins consisting of unmodified or peptides with minor modifications. Furthermore, Class II bacteriocins are subdivided into four subclasses: pediocin-like bacteriocins (class IIa), two-peptide bacteriocins (class IIb), cyclic bacteriocins (IIc) and linear non-pediocin-like bacteriocins (class IId) (4). Bacteriocins produced by LAB have been intensively explored from a fundamental perspective, for their potential applications as food preservatives and, more recently, in veterinary and human medicine as possible alternative to antibiotics.

Positive properties of bacteriocins, which make them suitable for application in the food industry, they are inactive and non-toxic to eukaryotic cells and are sensitive to digestive proteases and so have little influence on gut microbiota. The application of bacteriocins (nisin and pediocin PA-1 are commercially available for food preservative uses) in the food industry provide many benefits such as the replacement of chemical preservatives or allowing the reduction of the intensity of heat treatment resulting in food that is more naturally preserved and with better sensorial and nutritional properties. Furthermore, bacteriocins are relatively thermostable and some of them can retain antimicrobial activity following pasteurization or sterilization. Also, some bacteriocins have a broad spectrum of antimicrobial

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activity, so they can be used in foods as an effective method for extending shelf life and to control food-borne pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes* (5, 6). *L. monocytogenes* is of particular concern since it is the causative agent of listeriosis (it can traverse the intestinal, placental and blood/brain barriers in humans) a relatively rare disease with high fatality rates (12%) in Europe and (25%) in the United States. Because of that, in the most European countries and in the United Stated, there are zero-tolerance standards for the *L. monocytogenes* in ready-to-eat (RTE) food (7–10).

Traditional fermented foods, such as cheeses produced from raw milk, are a rich ecological niche from which bacteriocin-producing LAB can be isolated (11). The indigenous LAB isolated from white brined cheeses from Serbia are good candidates for screening for antimicrobial substances as they are well adapted to the microbial environment`s in cheese and could therefore be the source of novel properties (12).

Aureocins are new group of leaderless class II bacteriocins with broad spectrum of activity firstly isolated from *Staphylococcus aureus*. Aureocins act bactericidally on sensitive cells causing rapid lysis (13–15). According to their structure they could be classified into two main groups: multi-peptide (aureocin 70 like) and one peptide aureocins (aureocin A53 like).

In a previous study, it was demonstrated that the crude extract obtained from cell free supernatant of the natural isolate *Lactococcus lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 inhibited growth, biofilm formation and reduced 24 h old biofilms of coagulase negative staphylococci and *Listeria monocytogenes* clinical isolates (16). The objective of this work was to purify and biochemically and genetically characterise the broad spectrum bacteriocin lactolisterin BU, produced by *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4.

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RESULTS

Localization of the genes coding bacteriocin(s) production

The activity spectrum of *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 is broad, inhibiting different strains of *Lactococcus, Lactobacillus, Enterococcus* species and some pathogenic strains (16). Standard biochemical methods confirmed the proteinaceus nature of antimicrobial agent as it was found to be sensitive to proteinase K and pronase E. In addition, it was active against B464, a man-PTS deletion mutant derivative of IL1403 (17) suggesting that man-PTS is not a receptor for its antilisterial activity. To determine the bacteriocin coding genes location, a plasmid curing assay was performed. It was interesting to note that three types of plasmid-cured derivatives were obtained which differed in activity spectrum and size of the inhibition zone in agar well diffusion assays. It was noticed that derivative BGBU1-4/2, showed a reduced zone of inhibition against *L. lactis* subsp. *lactis* BGMN1-596 and *L. monocytogenes* ATCC 19111 compared to the parental strain and were sensitive to the parental strain. Derivatives BGBU1-4/29 and BGBU1-4/8 did not show antimicrobial activity against BGMN1-596 and ATCC 19111 and were sensitive to the parental strain BGBU1-4 and derivative BGBU1-4/2. The plasmid profile analysis showed differences between parental strain BGBU1-4 and derivatives; derivative BGBU1-4/2 lost the plasmids pBU12 and pBU20, BGBU1-4/8 lost the smallest plasmid (pBU6) and pBU12, while in derivative BGBU1-4/29 three plasmids (pBU6, pBU12 and pBU20) were absent (Fig. 1). These results indicate that strain BGBU1-4 synthesizes at least two bacteriocins active against *Lactococcus* sp. and *L. monocytogenes* strains that are encoded on plasmids'. It was possible to conclude that there is a direct correlation between the presence/absence of plasmid pBU6 and bacteriocin activity and most likely that the operon for the synthesis of the second bacteriocin is located on the plasmid pBU12.

Purification and identification of bacteriocin(s)

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The bacteriocin(s) produced by strain BGBU1-4 were purified by RP-HPLC and the molecular mass of the active peptides determined by MALDI-TOF MS. The RP-HPLC chromatogram showed dominant peaks (Fig. 2) (fractions 30 and 37) that were active against *L. lactis* subsp. *lactis* BGMN1-596 and *L. monocytogenes* ATCC 19111. Mass spectrometry analysis determined the molecular mass of fraction 30 at 3642.62 Da and fraction 37 at 5160.94 Da. As fraction 37 was most active it was selected for further characterization.

N-terminal sequencing of protein fraction 37

N-terminal sequencing of the native peptide in fractions 30 and 37 failed most probably due to a blocked N terminus. N-terminal sequencing was challenged two times without success. To circumvent this, native peptides were digested with trypsin and N-terminal sequening of an internal fragments were done. Internal 1112.61 Da fragment of protein from fraction 37 revealed the amino acid sequence AVSWAWQH, which corresponds to lactolisterin BU residues 16-23 (Fig. 4), while N-terminal sequencing of peptides from fraction 30 failed two times and work was continued only on fraction 37 (lactolisterin BU). Lactolisterin BU is a leaderless peptide and consequently the N terminal amino acid is formylmethionine rather than methioine as there is no cleavage of the leader peptide. The formyl group of formylmethionine blocks the alpha carbon of the amino acid making it inaccessable to phenylisothiocyanate (PITC), the reagent used in N terminal sequencing (18). MALDI TOF MS was also used to confirm the presence of formylmethionine. Addition of a formyl group results in a 28 Da increase in mass which is in good agreement with the 29 Da mass difference oserved when the mass of the native peptide (5160.94 Da) was compared with the theoretical mass (5131.67 Da).

Heterologous expression of the bacteriocin in *Lactococcus lactis* subsp. *cremoris* MG7284

Plasmid curing indicates that genes for the synthesis and immunity of bacteriocins in strain *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 are located on plasmids. The smallest plasmid

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named pBU6 (6.2 kb) from strain BGBU1-4 was isolated and used for transformation of the bacteriocin non-producer *Lactococcus lactis* subsp. *cremoris* MG7284 strain. GM17 agar plates containing lactolisterin BU, with concentration of 1,34 µM, were used to select for bacteriocin-resistant transformants. Obtained transformants were designated MG7284/pBU6 and were used for further purification of lactolisterin BU. The plasmid profile analysis of transformants MG7284/pBU6 revealed that all transformants possess the smallest 6.2 kb plasmid and were found to be active against indicator strains BGMN1-596, ATCC 19111 and B464, confirming that man-PTS is not a receptor for its antilisterial activity (Fig. S1).

Analysis of plasmid pBU6

Plasmid pBU6 was sequenced in its entirety and submitted to the European Nucleotide Archive under accession No: LT629305.Sequence analysis of plasmid pBU6 revealed that it is a small rolling circle replicating (RCR) plasmid, which contains nine ORFs: *repB*, *lliBU*, *abcT*, *hyp1*, *hyp2*, *hyp3*, *mobC*, *relM*, *RnaseY* (Fig. 3, Table 2).

In silico analysis revealed that RepB protein shows high similarity with lactobacilli and lactococcal RepB proteins (*Lactobacillus fermentum* and *Lactococcus lactis*; WP_011117039.1, WP_032951507.1, respectively). The N-terminal sequencing results of the peptide digest of fraction 37 identified *lliBU* as the structural gene, encoding a 43-amino acid peptide, which is responsible for production of the bacteriocin, lactolisterin BU. Lactolisterin BU is leaderless bacteriocin rich in amino acids glycine and tryptophan (each 11.6%) with pI 10.16. Interestingly, this protein shows highest similarity with bacteriocin BHT-B from *Streptococcus ratti*, (63%, AAZ76605.1; (19)) and other aureocin A like bacteriocins (Fig. 4). Downstream of the *lliBU* gene ORF designated as *abcT* (212 aa) encodes a protein similar to the sugar ABC transporter ATP binding protein from *Streptococcus ratti* (62%, WP_003089811.1). One or more of the next three genes, *hyp1* (212aa), *hyp2* (169 aa), *hyp3* (92 aa), may encode a protein(s) that plays a role in producer immunity. The three genes

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mobC (127 aa), *relM* (333 aa) and *rnaseY* (169 aa) encode proteins similar to the Mobilization protein C, WP_010729194.1, Relaxase/mobilization nuclease, WP_010730379.1, and Ribonuclease Y, WP_017865030.1, respectively.

Biochemical characterisation of lactolisterin BU

Antimicrobial activity of purified lactolisterin BU was unchanged after heat treatment at 60°C, 80°C and 100°C for 15 and 30 min when compared with the control with MIC values of 0.67 µM against *L. lactis* subsp. *lactis* BGMN1-596 and 1.34 µM against *L. monocytogenes* ATCC19111.

The growth kinetics and antimicrobial activity of transformant MG7284/pBU6 is shown in Fig. 5. Detectable levels of bacteriocin activity were recorded after 2 h of growth at 30°C. Maximum bacteriocin activity of the MG7284/pBU6 was achieved at the end of exponential phase while activity was lower against indicator strain *Listeria monocytogenes* ATCC 19111 after 24 h growth (Fig. 5).

Purified lactolisterin BU was active against various Gram-positive bacteria in micromolar concentrations of MICs. Lactolisterin BU showed relatively strong activity against *Listeria monocytogenes* ATCC 19111 as the MIC value of the bacteriocin was 1.34 µM. In addition lactolisterin BU showed very strong antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Streptococcus pygoenes* A2941 and *Streptococcus pneumoniae* P156 (Table 3). It is interesting that purified lactolisterin BU exhibited antimicrobial activity against lactolisterin BU producers (parental strain BGBU1-4, cured derivative BGBU1-4/2 and transformants MG7284/pBU6) with MIC value of 1.34 µM (Table 3).

Mode of action of lactolisterin BU

Results of *L. monocytogenes* ATCC 19111 culture growth (started with different number of cells) in the presence of different concentrations of lactolisterin BU strongly indicated that the effect of lactolisterin BU on the growth is not growth phase dependent. The strongest lysis of

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bacteria was obtained when the cells were in the early logarithmic phase. With the increase in the number of cells, the effect of lactolisterin BU on stopping growth of sensitive bacteria is gradually decreased (when it was used in concentration of 1.34 µM, minimum inhibitory concentration), but at higher concentrations (4.02 and 13.4 µM) it exhibited strong inhibition of the growth of bacteria in all stages of growth (Fig. S2), indicating that it can be successfully used against pathogens and food contaminants that are in the stationary phase.

An attempt to isolate a lactolisterin BU resistant mutant

In our previous experiences in obtaining mutants resistant to bacteriocins we used two approaches: selection of spontaneous mutants and mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (20). We have successfully isolated mutants from both approaches but using mutagenesis we isolated greater number of mutants that showed greater diversity. First, we tried to isolate spontaneous mutants by spreading 500 µl of 10 times concentrated overnight cultures of sensitive strains on GM17 Petri dishes containing lactolisterin BU in concentrations of 1.34 µM and 2.68 µM (1 and 2 times MIC values). No mutant-resistant to lactolisterin BU was obtained indicating a possible different mechanism of action or that a mutation leading to resistance is lethal (target protein is essential). In order to confirm the impossibility of obtaining mutants resistant to lactolisterin BU, mutant banks of three sensitive strains (*L. lactis* subsp. *lactis* BGMN1-596, *L. lactis* subsp. *cremoris* MG7284 and *Enterococcus faecalis* BGZLS10-27) were constructed using N-methyl-N′-nitro-N-nitrosoguanidine (which increases the chance of getting more mutations in each of the genes) and used to isolate resistant mutants by spreading of aliquots on selective GM17 Petri dishes containing lactolisterin BU. We did not manage to select the mutant resistant to lactolisterin BU from the mutant banks after three attempts per each strain, confirming the treatment that we applied did not yield mutant resistant to lactolisterin BU, or it is very difficult to isolate such a mutant.

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DISCUSSION

Lactic acid bacteria are found in different ecological niches and, as a result of their efforts to adapt and survive, they produce various secondary metabolites among which are bacteriocins. Although, bacteriocins have been studied for almost seven decades, researchers still find them interesting due to their potential applications. In the last decade this field was broadened by their possible use instead of and/or in synergy with antibiotics to overcome the immense problem of increasein prevalence of antibiotic resistant bacteria (4). Natural isolates from traditionally prepared food products are a tremendous source of highly diverse, unique metabolites. These isolates come from harsh environments and contain genes that are usually lost in industrial strains. Lactococci commonly produce more than one bacteriocin and *Lactococcus lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 strain produces at least two bacteriocins (21–23). Crude extract from cell free supernatant of the strain BGBU1-4 demonstrated growth inhibition, and reduction of 24 h old biofilms formed by clinical isolates of *Listeria monocytogenes* and coagulase negative *Staphylococcus* sp., while prevention of biofilm formation was demonstrated for *L. mocytogenes* clinical isolates (16). Genes for bacteriocin lactolisterin BU production were plasmid pBU6 located which is not unusual (22–25), but plasmid cured derivatives suggest that broad spectrum antimicrobial activity of strain BGBU1-4 is a consequence of production of at least two bacteriocins. Characterization of the anti-listerial bacteriocin purified by HPLC revealed that ORF *lliBU* on pBU6 encodes the lactolisterin BU structural gene. In addition, sequence analysis of lactolisterin BU, a 43-amino acid peptide, shows the highest similarity with BHT-B, a Class II bacteriocin from *Streptococcus ratti*. Although lactolisterin BU shows some characteristics of Class IIa (anti-listerial activity and absence of unusual amino acids) bacteriocins it does not possess the highly conserved motif T-G-N-G-V/L generally found in “pediocin-like” bacteriocins with antilisterial activity and so cannot be classified as a Class IIa bacteriocin (26–28). Therefore it

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is classified as a Class IId leaderless bacteriocin and can be compared to LsbB, Lacticin Q, Lacticin Z, aureocins A70 and A53, bacteriocins known to be synthesized and exported without a leader sequence (14, 15, 23, 29, 30). A comparative analysis of the amino acid sequences has shown that lactolisterin BU shares a conserved region AKYGxKAV with the majority of known aureocin A53 like bacteriocins (Fig. 4). It can be assumed, considering the variation in length and primary structure, that the region AKYGxKAV is responsible for the activity of aureocin A53 like bacteriocins. It is interesting that lactolisterin BU shows higher identity with BHT-B bacteriocin from *Streptococcus ratti*, (63%) than with lacticin Q and lacticin Z (33.9%) isolated from lactococci. Antilisterial activity of lactolisterin BU is not mediated by interaction with man-PTS, like in other Class II bacteriocins, as it showed antimicrobial activity on mutant B464 (17).

Biochemical characterization showed that lactolisterin BU, like other bacteriocins, is sensitive to proteolytic enzymes, relatively thermostable, and has maximum production in early stationary phase (31–33). Lactolisterin BU has potential as a food preservative due to its strong antimicrobial activity (active in micromolar concentrations) against many species of Gram-positive bacteria, including important food spoilage and food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus* sp. and *Streptococcus* sp.. Additionally, since production of many food products involves exposure to high temperature, the relative thermostability of lactolisterin BU is another desirable feature for its application in the food industry. The most desirable characteristic of lactolisterin BU for its use in controlling contaminants of food or pathogens is the inability to induce resistance that is most likely the result of a specific mechanism of action or an essential target molecule. Genes for bacteriocin production are often plasmid located enabling horizontal gene transfer between genera and it is expected that rearrangements during transfer results in novel peptides. It is assumed that a similar scenario happened with lactolisterin BU. The presence of a highly

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conserved region between lactococcal bacteriocin lactolisterin BU, bacteriocin BHT-B from *Streptococcus ratti*, aureocin A53 from *Staphylococcus aureus* and aureocin A53 like bacteriocin from *Corynebacterium jeikeium* indicates a common origin of the bacteriocin operon. It is interesting that the homologous bacteriocin operon is present in such a wide variety of genera, which indicates that bacteriocin production confers an advantage to the carrier. The greater similarity between lactolisterin BU and aureocins from other bacteria than with lactococcal (lacticin Q and Z) indicates a different evolutionary pathway of these lactococcal bacteriocins.

This work provides insight into a new and unusual Class II lactococcal bacteriocin, lactolisterin BU. Genetic and biochemical characteristics, activity spectrum, protease sensitivity, thermostability and inability or very rare occurrence of resistance recommend lactolisterin BU as good candidate for a safe, cheap and natural food preservative. Further experiments on lactolisterin BU in preventing *L. monocytogenes* development in products obtained from raw milk are ongoing.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. The bacteriocin producer *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 was isolated from a three day old traditional semi-hard cheese made from mixed cow (20%) and sheep (80%) milk (34). The cheese was produced without the use of starter cultures in a household in the village of Buzina, located on the mountain Beljanica in eastern Serbia. Lactococcal strains were grown in M17 medium (Merck GmbH, Darmstadt, Germany) supplemented with D-glucose (0.5% w/v) (GM17) at 30°C. *Lactobacillu*s strains were grown in MRS medium (Merck GmbH, Darmstadt, Germany). Non-lactococcal indicator strains were grown aerobically in Luria-Bertani (LB) broth at 37°C. *Streptococcus* strains were grown in Brain Heart Infusion (BHI) medium

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(Oxoid, Basingstone, Hampshire, England) at 37°C and an atmosphere of 5% CO_2. Solid medium and soft-agar were made by adding 1.5% or 0.7% (w/v) agar (Torlak, Belgrade, Serbia), to the liquid media, respectively.

Spectrum and kinetics of bacteriocin activity

The agar well diffusion assay was used to determine the antibacterial spectrum of the strain BGBU1-4 (33). Each indicator strain was inoculated into appropriate soft-agar, and wells (diameter 5 mm) were made in the plate. The wells were filled with 50 µL of sample and plates were incubated under appropriate conditions for the respective indicator strain (Table 2). After 24 h of incubation, plates were examined for the presence of inhibition zones. A clear zone of inhibition around the wells was taken as evidence of bacteriocin production. To monitor kinetics of bacteriocin production/activity 100 mL of fresh preheated GM17 broth was inoculated with overnight culture (1% v/v) and incubated at 30°C. Samples were taken at 0, 2, 4, 6, 8, 10, 12, and 24 h. Bacteriocin activity was determined by area zone of inhibition. *L. monocytogenes* ATCC 19111 and *L. lactis* subsp. *lactis* BGMN1-596 were used as indicator strains.

Genetic characterization

Plasmid curing experiments

Plasmid curing assays were done by growing the bacterial cells of BGBU1-4 strain in the presence of novobiocin at sub-lethal temperatures as described previously (35). Preheated GM17 broth (42°C) containing novobiocin (5 µg/mL) was inoculated with 10^3 cells per mL. After 2 h of incubation, the cells were collected by centrifugation and resuspended in the same volume of fresh preheated novobiocin containing GM17 broth to avoid a bacteriocin-killing effect on the cured cells. This step was repeated four times and end point aliquots (0.1 mL) were plated onto GM17 agar plates, which were then incubated at 30°C for 48 h.

Molecular methods

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Derivatives obtained following plasmid curing experiments, and transformants were confirmed using pulsed field gel electrophoresis (PFGE) as described previously by Kojic et al., 2006 (23). For isolation of total DNA from lactococci, a modified version of the method described by Hopwood et al., 1985 (36) was used. Plasmid DNA from lactococci was isolated by the modified method previously described by O`Sullivan and Klaenhamer, 1993 (37). Plasmids were introduced into lactococci by electroporation using an Electroporator (Eppendorf, Hamburg, Germany) (38). Plasmid DNA was sequenced by the Macrogen Sequencing Service (Macrogen Europe, Amsterdam, The Netherlands). Nucleotide sequences were analysed using BLAST algorithm. The functions of the proteins encoded by the pBU6 plasmid were attributed on the basis of homology with known proteins by using BLAST comparison with Entrez protein blast.

Purification of the bacteriocin

Lactolisterin BU was purified from the cells according to the method of Rea et al., 2007 (39) with the following modifications. Briefly, the cell pellet from a 2 litre culture grown in TY broth was resuspended in 250 mL of 70% (v/v) 2-propanol, 0.1% (v/v) TFA/L of broth and stirred at room temperature for 3-4 hours. Sample was centrifuged as at 8280 *g* for 20 minutes and cell supernatant retained for purification. The 2-propanol was evaporated using a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) and the sample applied to a 5 g (20 mL) Strata C18-E SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 40 mL of 30% (v/v) ethanol, and the bacteriocin was eluted with 40 mL of 70% (v/v) 2-propanol, 0.1% (v/v) TFA. An aliquot of the cell C18 SPE 70% 2-propanol, 0.1% TFA eluent was concentrated using rotary evaporation before separation of the peptides using RP-HPLC. Aliquots of approximately 4 mL were applied to a Phenomenex (Phenomenex, Macclesfield Cheshire, UK) Proteo Jupiter (RP)-HPLC column (250 × 10.0 mm, 4μ, 90Å) previously equilibrated with 25% acetonitrile, 0.1% TFA. Peptides

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were eluted in a gradient of 30% acetonitrile containing 0.1% TFA to 70% acetonitrile containing 0.1% TFA over 40 minutes where buffer A is Milli Q water containing 0.1% TFA and buffer B is 90% acetonitrile containing 0.1% TFA and the flow rate was 2.5 mL/min. Fractions were collected at 1 minute intervals and assayed on *Lactococcus lactis* BGMN1-596 and *Listeria monocytogenes* ATCC 19111indicator plates.

MALDI TOF Mass spectrometry was performed on fractions exhibiting positive inhibitory activity, using an Axima TOF^2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK), as described by Mills et al., 2011 (40).

N-terminal sequencing of protein from fractions 30 and 37

Protein fractions 30 and 37 that showed antimicrobial activity were sent to Department of Molecular and Biomedical Sciences “Jozef Stefan” Institute (Ljubljana, Slovenia) for N-terminal sequencing by Edman degradation. N-terminal sequencing of the native peptides failed due to the presence of an N terminal formyl methionine so peptides were digested with trypsin and trypsin fragments were sequenced on the second attempt.

Determination of minimal inhibitory concentrations (MICs)

The minimal inhibitory concentration of the lactolisterin BU was determined using the broth microdilution method proposed by Steinberg et al., 1997 (41). The microdilution testing assay used a mixture of the indicator strains (Table 1 and Table 3) and increasing concentrations of bacteriocin, lactolisterin BU. Microdilution testing, with in-house prepared panels was performed following the Clinical and Laboratory Standards Institute’s Performance Standards for Antimicrobial Susceptibility Testing (Twenty-Fourth Informational Supplement. CLSI document M100-S24). Indicator strains were diluted to 0.5 McFarland units from which 20 µL were distributed to wells of a clear 96-well flat bottom microtiter plate. Concentration of pure lactolisterin BU was determined by spectroscopic method using theoretical extinction coefficient calculated from the peptide sequence as described by Blanusa et al., 2007 (42).

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Lactolisterin BU (47 µM) was two-fold serially diluted to give a dilution series from 43 µM to 0.67 µM. The microtiter plates were incubated under appropriate conditions for 24 h, and the optical densities at 595 nm (OD_{595}) were recorded at 30 min intervals (Infinite M200pro, Tecan, Switzerland). Values obtained were used to illustrate the antimicrobial activity of the bacteriocin lactolisterin BU. Control wells contained appropriate medium (blanks) and untreated culture. All experiments were done in triplicate.

Effect of temperature treatment on lactolisterin BU activity

Water dissolved purified lactolisterin BU (concentration of 47 µM), was incubated at 60°C, 80°C and 100°C for 15 min and 30 min. After treatments, antimicrobial activity was determined using the broth microdilution method proposed by Steinberg et al., 1997 (41) as described above. The indicator strains were *L. monocytogenes* ATCC 19111 and *L. lactis* subsp. *lactis* BGMN1-596; untreated purified bacteriocin was used as a control. All experiments were done in triplicate.

Mode of action of lactolisterin BU

To analyze the effect of lactolisterin BU on the growth of the sensitive strain, purified lactolisterin BU (three different concentrations were used; 1.34 µM – minimal inhibitory concentration, 4.02 µM –three times higher than MIC, and 13.4 µM – ten times higher concentration than MIC) was added to the cultures of *L. monocytogenes* ATCC 19111 inoculated with different number of bacteria (3×10^7, 3.3×10^8 and 2×10^9 cells/mL) in microtiter plates. Before the addition of bacteriocin, diluted bacterial cultures were incubated for 1 hour at the optimal growth temperature to refresh the cells. The bacterial growth was monitored by measurement of optical densities at 595 nm (OD_{595}) that were recorded at 30 min intervals (Infinite M200pro, Tecan, Switzerland) and by determination of viable bacterial cells (CFU) at every hour of growth. Control wells contained appropriate medium (blanks) and untreated cultures. All experiments were done in triplicate.

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Mutagenesis of sensitive strains with N-methyl-N′-nitro-N-nitrosoguanidine.

Cells from middle logarithmic growth phase (OD_{600} ~ 0.6-1) of sensitive strains (*L. lactis* subsp. *lactis* BGMN1-596, *L. lactis* subsp. *cremoris* MG7284 and *Enterococcus faecalis* BGZLS10-27) were harvested by centrifugation at 10 000 *g* for 10 min at 4°C and washed two times in the same volume of 100 mM sodium phosphate buffer (pH 7). Ten times concentrated cells in phosphate buffer were exposed to different concentrations of N-methyl-N′-nitro-N-nitrosoguanidine (0, 25, 50, 100 and 200 µg/mL in phosphate buffer) for 1 h at 30°C in dark. After treatment cells were washed two times with 10 times volume of phosphate buffer and finally resuspended in the same volume of GM17. Cultures were grown for 1 h at 30°C in dark in order to recover cells and cell survive was determined by plating of 10 times dilutions on GM17 plates and incubation at 30°C for two days. Stabilised mutated cells were stored at -80°C by adding glycerol (final concentration 15%) until use.

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FIGURE LEGENDS

Figure 1. Plasmid profile analysis of parental strain *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 and its cured derivatives on 1% agarose gel. M - the upper part of GeneRuler 1kb DNA ladder Thermo Fisher Scientific (from top to bottom: 10 kb, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.75 kb), chrom – indicates position of chromosomal DNA. Only plasmids of the selected cured derivatives are shown in the figure, taken from the different positions of the gel.

Figure 2. Reverse-phase high performance liquid chromatography chromatogram (A) (RP-HPLC) and Matrix laser desorption ionization time of flight (MALDI-TOF) mass spectrometry data (B, C). Arrow indicates location of the antimicrobial peptide.

Figure 3. Circular restriction map of plasmid pBU6. Only relevant restriction sites and their positions are indicated; unique restriction sites are indicated by bold letters. The position and orientation of the genes are indicated by arrows.

Figure 4. Alignment of lactolisterin BU (SDR48784) amino acid sequence with homologous bacteriocins: lacticin Q from *Lactococcus lactis* QU5 (BAF57910.1), lacticin Z from *Lactococcus lactis* QU14 (BAF75975.1), bacteriocin BHT-B from *Streptococcus ratti* (DQ145753.1), aureocin A53 from *Staphylococcus aureus* (AAN71834) and aureocin A53 like bacteriocin from *Corynebacterium jeikeium* (WP_010976360). Highlighted residues indicate conservation in at least five of the peptide sequences while an asterisk indicates completely conserved residues. 'x' corresponds to unconserved residues, periods indicate amino acids belonging to similar groups, and colons indicate amino acids belonging to the same group.

Figure 5. Lactolisterin BU levels in culture relative to the cell density of *Lactococcus lactis* subsp. *cremoris* MG7284/pBU6 tested on *L. monocytogenes* ATCC 19111. Filled squares represent bacterial growth measured by colony forming units (CFU) circles indicate

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corresponding bacteriocin activity determined by area of zone inhibition. Error bars represent standard deviations of three independent experiments.

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Table 1. Strains used in this study

Strains or plasmids	Relevant characteristic(s)	Source or reference
Lactococcus lactis		
BGBU1-4	Bacteriocin producer (lactolisterin BU)	(34)
BGBU1-4/2	Derivate of BGBU1-4; Bac^{+}; Bac^{s}	This work
BGBU1-4/8	Derivate of BGBU1-4; Bac^{+}; Bac^{s}	This work
BGBU1-4/29	Derivate of BGBU1-4; Bac^{-}; Bac^{s}	This work
BGMN1-596	Bac^{-}, Bac^{s}	(20)
MG7284	Bac^{-}, Bac^{s}	(43)
MG7284/pBU6	MG7284 transformed with pBU6 plasmid (producer of lactolisterin BU)	This work
B464	Man-PTS deletion mutant of strain IL1403	(44)
Lactobacillus casei BGHN14	Bac^{-}, Bac^{s}	(45)
Listeria monocytogenes		ATCC19111
Staphylococcus aureus		ATCC 25923
Enterococcus faecalis		ATCC 29212
Enterococcus faecalis BGZLS10-27		(46)
Bacillus cereus		ATCC 11778
Bacillus subtilis subsp. *subtilis*		ATCC23857
Streptococcus pyogenes A2941		Pasteur laboratory, Belgrade
Streptococcus pneumonia P156		Pasteur laboratory, Belgrade
Escherichia coli H7:O157		ATCC 35150
Pseudomonas aeruginosa		ATCC 27853
Salmonella Typhimurium		ATCC 14028
Salmonella Enteritidis		ATCC 13076

Bac^{+} = bacteriocin producer, Bac^{-} = non-bacteriocin producer, Bac^{s} = sensitivity to lactolisterin BU; ATCC= American Type Culture Collection, Manassas, VA, USA

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Table 2. Results of BLAST comparison of proteins encoded by plasmid pBU6 with Entrez protein database. The number of amino acids and the position from the beginning of the sequence of plasmid pBU6 are given for each ORF.

orf **(No. of amino acids) position***	**Proteins with the highest identity, accession number**	**Predicted domain(s) or superfamily in encoded ORF**	**Organism**	**Amino acid identity (%)**
RepB (278 aa) 80-916	RepB, WP_011117039.1 RepB, WP_032951507.1	Rep_3 (pfam01051)	*Lactobacillus fermentum* *Lactococcus lactis*	90% 85%
LliBU (43 aa) 1034-1165	Aureocin-like bacteriocin, AAZ76605.1	Bacteriocin_Iii (pfam11758)	*Streptococcus ratti*	63%
AbcT (212 aa) 1230-1868	Sugar ABC transporter ATP-binding protein, WP_003089811.1	ABC_DR_subfamily_A (CD03230)	*Streptococcus ratti*	62%
Hyp1 (212 aa) 1861-2449	Hypothetical protein, WP_003089809.1	/	*Streptococcus ratti*	42%
Hyp2 (169 aa) 2503-3012	Hypothetical protein, AAZ76608.1	/	*Streptococcus ratti*	43%
Hyp3 (92 aa) 3531-3809	Hypothetical protein WP_027822861.1	/	*Lactobacillus plantarum*	51%
MobC (127 aa) 3829-4212	Mobilization protein C, WP_010729194.1 Hypothetical protein WP_011117545.1	MobC (pfam05713) /	*Enterococcus faecium* *Enterococcus faecalis*	41% 55%
RelM (333 aa) 4194-5195	Relaxase/mobilization nuclease, WP_010730379.1	Relaxase (pfam03432)	*Enterococcus faecium*	51%
RNaseY (169 aa) 5219-5728	Ribonuclease Y, WP_017865030.1	Phosphodiesterase (PRK12704)	*Lactococcus lactis*	29%

aa = amino acid, * = position of gene on plasmid pBU6

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Table 3. Antimicrobial spectra of purified lactolisterin BU

Indicator strains	MIC (μM)
Lactococcus lactis subsp. *lactis* BGMN1-596	0.67
Lactococcus lactis subsp. *lactis* BGBU1-4	1.34
Lactococcus lactis subsp. *lactis* BGBU1-4/2	1.34
Lactococcus lactis subsp. *cremoris* MG7284/pBU6	1.34
Lactobacillus casei BGHN14	0.67
Listeria monocytogenes ATCC 19111	1.34
Staphylococcus aureus ATCC 25923	0.67
Enterococcus faecalis ATCC 29212	1.34
Enterococcus faecalis BGZLS10-27	1.34
Bacillus subtilis subsp. *subtilis* ATCC23857	5.375
Bacillus cereus ATCC 11778	5.375
Streptococcus pyogenes	0.67
Streptococcus pnumoniae	0.67
Escherichia coli H7:O157 ATCC 35150	N.A.
Pseudomonas aeruginosa ATCC 27853	N.A.
Salmonella Typhimurium ATCC 14028	N.A.
Salmonella Enteritidis ATCC 13076	N.A.

N.A.- no activity

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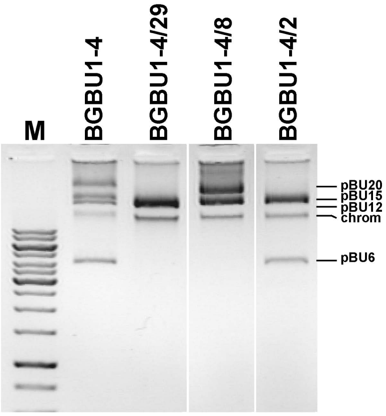

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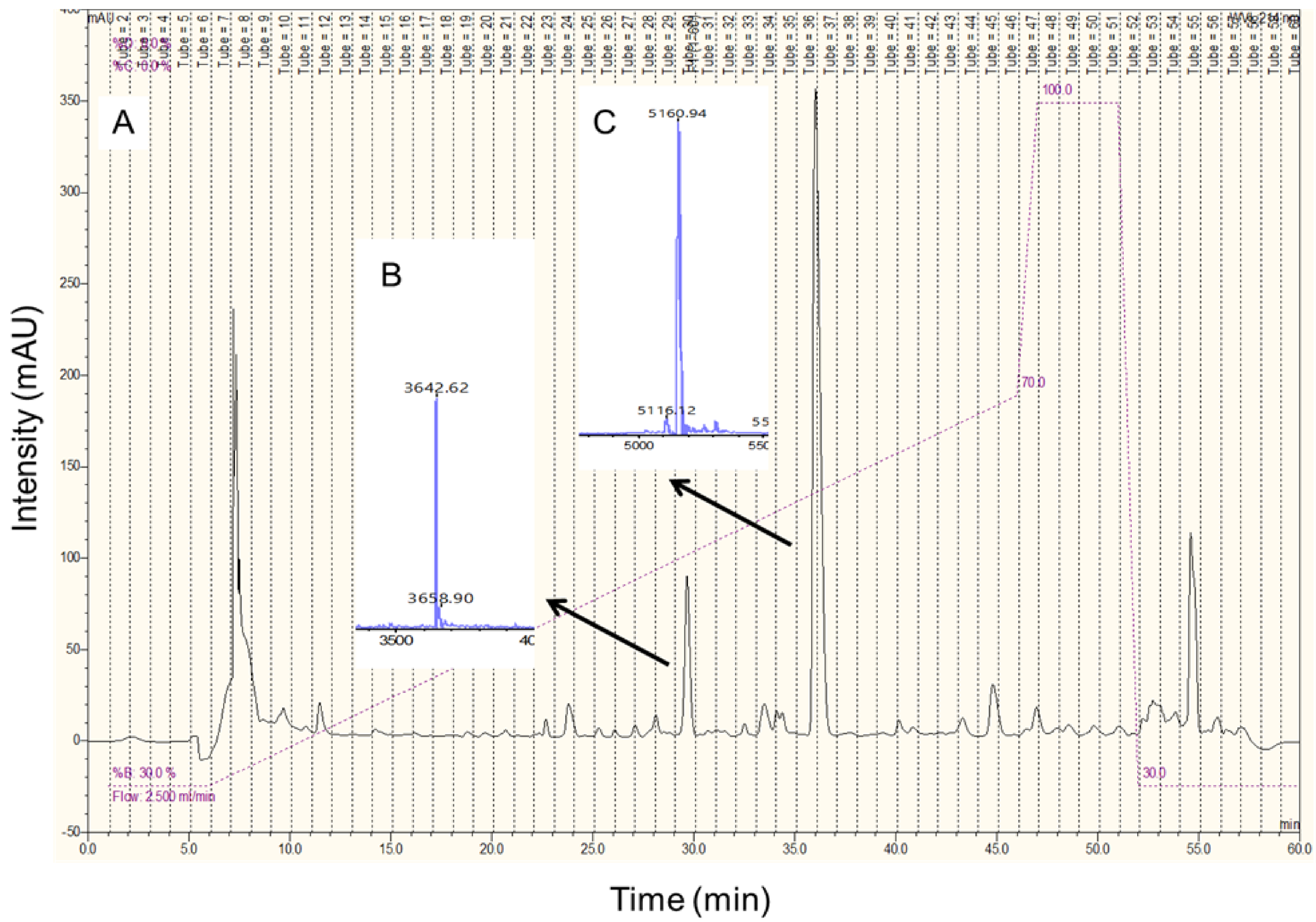

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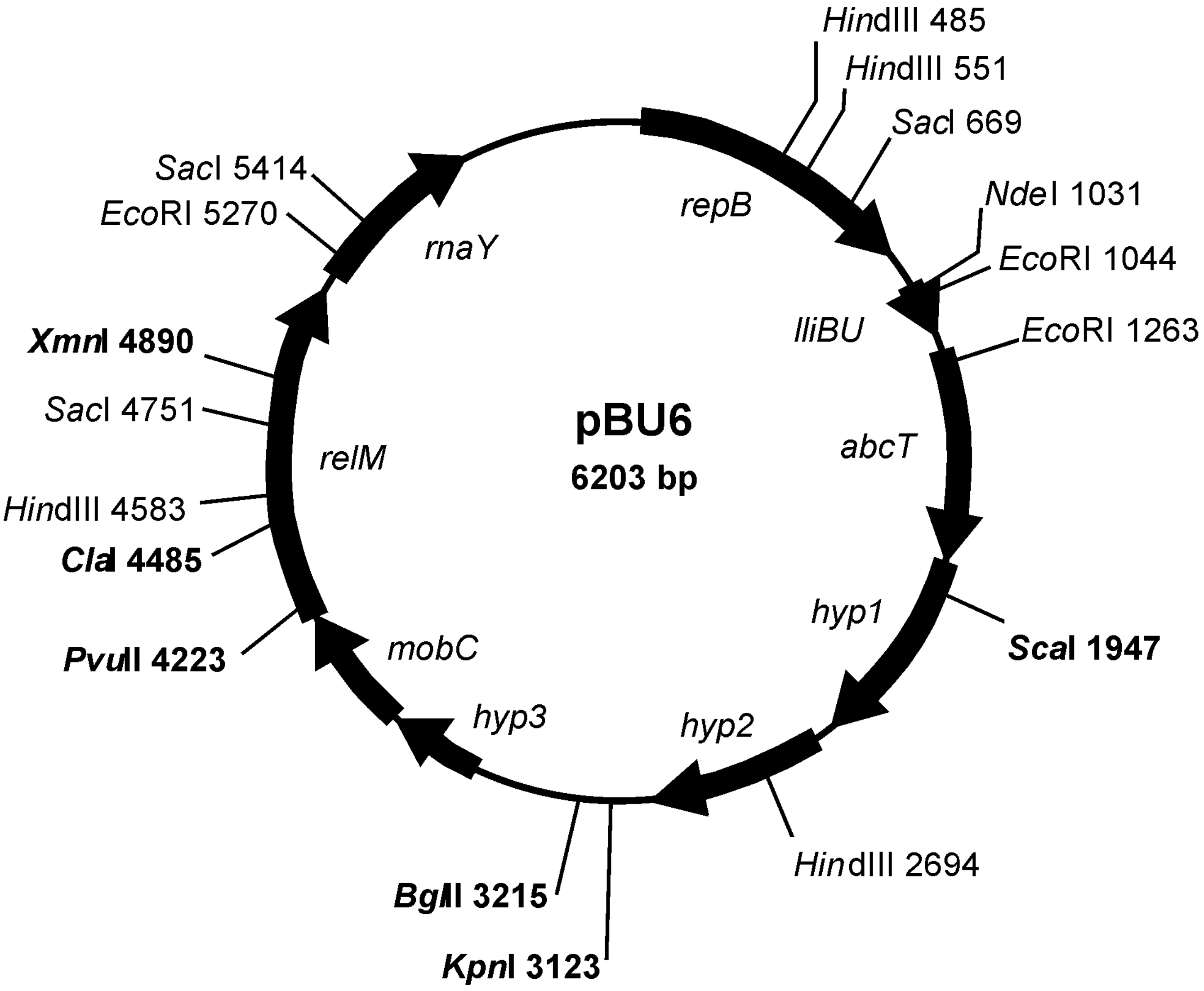

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Lactolisterin BU       --MWGRILGTVAKYGPKAVSWAWQHKWE-LI--NMG---DLAFRYIQRIWG--
Lacticin Q             MAGFLKVVQLLAKYGSKAVQWAWANKGKILDWLNAGQAIDWVVSKIKQILGIK
Lacticin Z             MAGFLKVVQILAKYGSKAVQWAWANKGKILDWINAGQAIDWVVEKIKQILGIK
Bacteriocin BHT-B      --MWGRILAFVAKYGTKAVQWAWKNKWFLL---SLG---EAVFDYIRSIWGG-
Aureocin A53           -MSWLNFLKYIAKYGKKAVSAAWKYKGKVLEWLNVGPTLEWVWQKLKKIAGL-
Aureocin A53 like      MAGFLKVVKAVAKYGSKAVKWCWDNKGKILEWLNIGMAVDWIVEQVRKIVGA-
                          : ..:  :**** ***. .*. *  :*   ::*   : .   :: * *

Identity consensus     xxxxxxxxxxxAKYGxKAVxxxWxxKxxxLxxxxxGxxxxxxxxxxxxIxGxx
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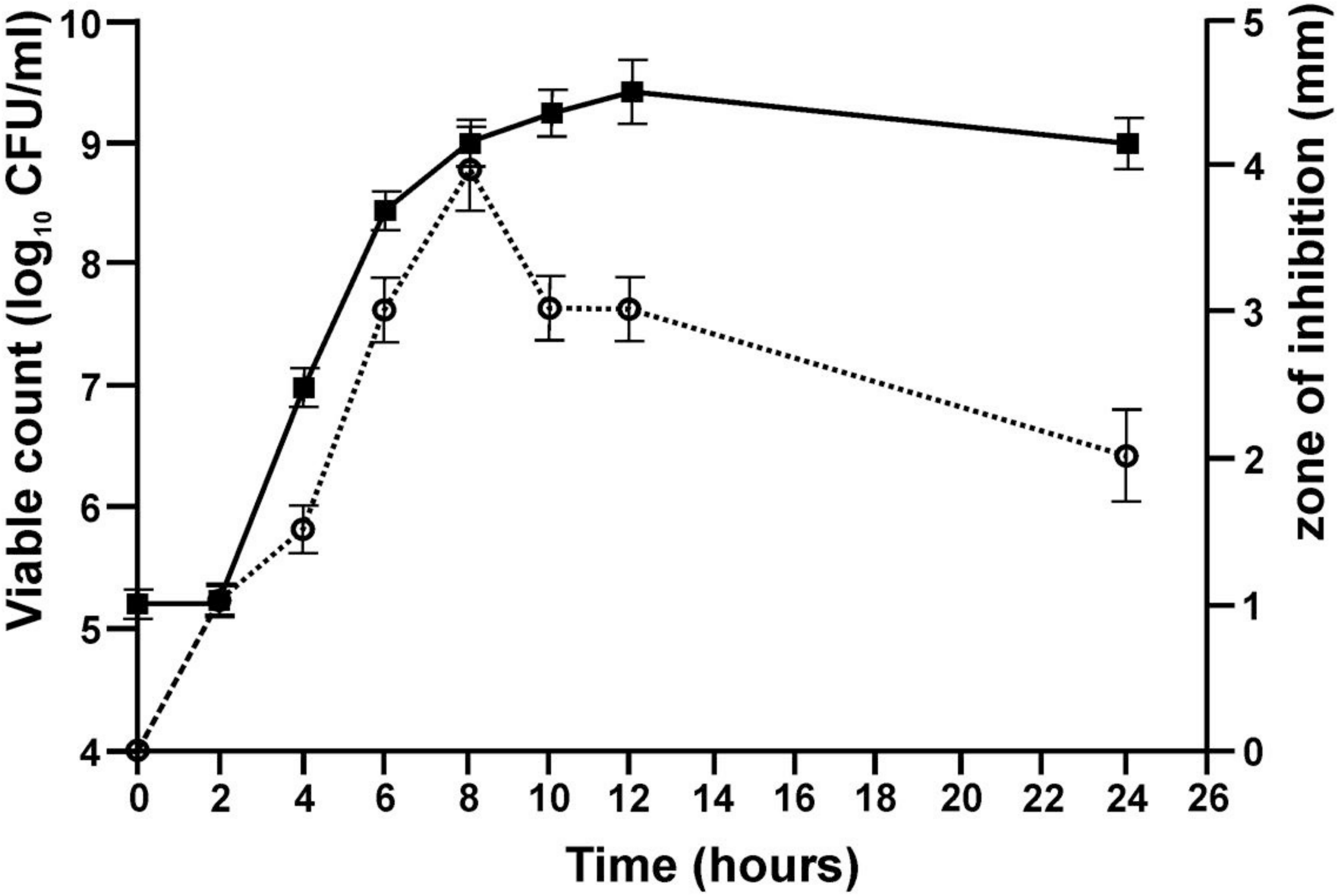

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