

Inhibition of Fungi and Gram-Negative Bacteria by Bacteriocin BacTN635 Produced by *Lactobacillus plantarum* sp. TN635

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Abstract The aim of this study was to evaluate 54 lactic acid bacteria (LAB) strains isolated from meat, fermented vegetables and dairy products for their capacity to produce antimicrobial activities against several bacteria and fungi. The strain designed TN635 has been selected for advanced studies. The supernatant culture of this strain inhibits the growth of all tested pathogenic including the four Gram-negative bacteria (*Salmonella enterica* ATCC43972, *Pseudomonas aeruginosa* ATCC 49189, *Hafnia* sp. and *Serratia* sp.) and the pathogenic fungus *Candida tropicalis* R2 CIP203. Based on the nucleotide sequence of the 16S rRNA gene of the strain TN635 (1,540 pb accession no FN252881) and the phylogenetic analysis, we propose the assignment of our new isolate bacterium as *Lactobacillus plantarum* sp. TN635 strain. Its antimicrobial compound was determined as a proteinaceous substance, stable to heat and to treatment with surfactants and organic solvents. Highest antimicrobial activity was found between pH 3 and 11 with an optimum at pH=7. The BacTN635 was purified to homogeneity by a four-step protocol involving ammonium sulfate precipitation, centrifugal microconcentrators with a 10-kDa membrane cutoff, gel filtration Sephadex G-25, and C18 reverse-phase HPLC. SDS-PAGE analysis of the purified BacTN635, revealed a single band with an estimated molecular mass of approximately 4 kDa. The maximum bacteriocin production (5,000 AU/ml) was recorded after a 16-h incubation in Man, Rogosa, and Sharpe (MRS) medium at 30°C. The mode of

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action of the partial purified BacTN635 was identified as bactericidal against *Listeria ivanovii* BUG 496 and as fungistatic against *C. tropicalis* R2 CIP203.

Keywords *Lactobacillus plantarum* sp. TN635 · Identification · BacTN635 · Purification · Broad inhibitory spectrum

Introduction

During the last two decades, with the misuse use of existing antibiotics in human and veterinary medicine and in agriculture, the effectiveness of currently available antibiotics is decreasing due to the increasing number of resistant pathogenic strains. This is presently an urgent focus of research and it becomes inevitable to discover new antimicrobial agents for combating such a problem. Numerous antibacterial agents are now being considered, such as bacteriophage [1], probiotic bacteria [2], antimicrobial peptides [3], and bacteriocins [4]. Bacteriocins are ribosomally synthesized peptides with a narrower spectrum of antimicrobial activity than most antibiotics [5]. Their antimicrobial activity has been found more effective against closely related strains [6]. Actually, attention is turning to the potential application of bacteriocins in the protection of human health [7], in agriculture [8], and in food preservation [9]. Although bacteriocins are produced by many Gram-positive and Gram-negative bacteria, those produced by lactic acid bacteria (LAB) are of particular interest because they are known as “good bacteria” and can be used as natural preservatives in food industry [10]. During the last few years, a large number of new LAB bacteriocins have been identified and characterized. However, only few have been described to possess activities against Gram-negative bacteria, e.g., plantaricin 35d produced by *Lactobacillus plantarum* [11], bacteriocin ST34BR produced by *L. lactis* subsp. *Lactis* [12], bacteriocins ST26MS and ST28MS produced by *L. plantarum* [13] and bacteriocin AMA-K produced by *L. plantarum* AMA-K [14].

During our search program for bioactive compounds from LAB, a new bacterium called TN635 was selected. This paper describes the selection, identification, and phylogenetic determination of this bacterium. The purification and the characterization of a bacteriocin-like substance called BacTN635, from the supernatant culture of this new strain are also reported.

Materials and Methods

Bacterial Strains and Plasmids

The TN635 strain was selected as a producer of potent antimicrobial activities and was used as the source of chromosomal DNA to amplify the 16S rRNA gene. *E. coli* TOP10 (Invitrogen), F-*mcrA* $\Delta(mrr-hsdRMS-mcrBC)$ ϕ 80*lacZ* Δ M15 Δ *lacX*74 *deoR* *recA1* *araD*139 $\Delta(ara-leu)$ 7697 *galU* *galK* *rpsL* *endA1* *nupG*, and *E. coli* DH5 α [15] were used as host strains. Bacterial strains: Gram-positive bacteria (*Micrococcus luteus* LB 14110, *Staphylococcus aureus* ATCC 6538, *Listeria ivanovii* BUG 496, *Enterococcus faecalis* JH 2-2 and *Enterococcus faecium* ENSAIA 631), Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Salmonella enterica* ATCC43972, *Pseudomonas aeruginosa* ATCC 49189, *Hafnia* sp. and *Serratia* sp.), and lactic acid bacteria: (*Lactobacillus casei* DSM 20011, *Lactobacillus sakei* 2525, *Lactobacillus delbrueckii* DSM 20081, *Lactococcus lactis* ATCC

11454 and *Lactococcus lactis* subsp. *cremoris* DSM 11603) were used as indicator microorganisms for the antibacterial activity assays. Antifungal activity was determined against the *Fusarium* sp. and *Candida tropicalis* R2 CIP203.

pIJ2925 [16] derivative of pUC18 and pCR-Blunt vector (Invitrogen) *Col* E1 origin (pUC-derived) Km^R were used as the cloning vectors. pSS1, derivative of pCR-Blunt vector carrying a 1,540-bp DNA fragment corresponding to the whole 16S rRNA gene of the TN635 strain (this work).

Culture Conditions

E. coli DH5 α was grown on Luria Bertani (LB) plates supplemented with ampicillin (50 $\mu\text{g}/\text{ml}$) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (40 $\mu\text{g}/\text{ml}$) when appropriate [17]. Transformation of *E. coli* DH5 α with pIJ2925 derivatives was carried out according to Hanahan [15]. Growth and transformation of TOP10 *E. coli* strain with the pCR-Blunt vector derivative were carried out according to the manufacturer's instructions (Invitrogen). For determination of antibacterial activities, indicator microorganisms were grown overnight in LB medium in aerobic conditions at 30°C for *Lb. casei* DSM 20011, *Lb. sakei* 2525, *Lc. lactis* ATCC 11454, *Lc. lactis* subsp. *cremoris* DSM 11603, *L. ivanovii* BUG 496, *S. enterica* ATCC43972, *P. aeruginosa* ATCC 49189, and *M. luteus* LB 14110, in aerobic conditions at 37°C for *E. coli* ATCC 8739 and *S. aureus* ATCC 6538, in anaerobic conditions at 30°C for *E. faecalis* JH 2-2, *E. faecium* ENSAIA 631, *Hafnia* sp and *Serratia* sp, and in anaerobic conditions at 37°C for *Lb. delbrueckii* DSM 20081. For antifungal activities determination, *Fusarium* sp. was grown in potato dextrose agar (PDA) for 7 days at 30°C. Spores were collected in sterile distilled water and then concentrated to produce a suspension with approximately 10^4 spores/ml. *C. tropicalis* R2 CIP203 was grown in YP10 medium (10 g/l yeast extract, 10 g/l peptone, 100 g/l glucose, 15 ml of 2 g/l adenine solution) at 30°C for 24 h in an orbital incubator with shaking at 200 rpm. The TN635 strain was maintained as frozen stocks at -80°C in Man, Rogosa, and Sharpe (MRS) broth containing 20% (v/v) glycerol. Before experimental use, the cultures were propagated twice in MRS medium at 30°C for 12 h and the transfer inoculum was 1% (v/v).

DNA Isolation and Manipulation

Total DNA preparation from TN635 strain was carried out according to the method of Leenhouts et al. [18] with slight modifications as described by Lee et al. [19]. Small-scale plasmid preparations from *E. coli* were performed as described by Sambrook et al. [17]. Digestion with restriction endonucleases, separation of DNA fragments by agarose gels electrophoresis, dephosphorylation with alkaline calf intestinal phosphatase, ligation of DNA fragments, and transformation of *E. coli* were performed according to Sambrook et al. [17].

PCR Amplification of the 16S rRNA Gene of TN635 Strain

PCR amplification of the 16S rRNA gene of TN635 strain was performed using two specific primers P1: 5'-AGAGTTTGATCCTGGCTCAG-3' and P2: 5'-ATGGCTCAGGACGAACGCT-3'. Approximately 200 ng genomic template DNA was used with 150 pmol of each primer per 50 μl reaction volume. Amplification was performed in an automated thermocycler (Perkin Elmer) using 1 U Pfu DNA polymerase (Stratagene) according to the following amplification profile: 94°C (3 min) followed by 40 cycles of denaturation at 94°C (30 s), annealing at 60°C (1 min), and extension at 72°C (3 min).

DNA Sequencing and Analysis

Nucleotide sequence of the whole 16S rRNA gene of TN635 strain was determined on both strands by an automated 3100 Genetic Analyser (Applied Biosystems) using specific primers. Homology search was performed using Blast Search algorithm. The nucleotide sequence of the whole 16S rRNA gene (1,540 pb) of TN635 strain has been assigned GenBank (EMBL) under accession number FN252881. Multiple sequence alignment was carried out using CLUSTAL W [20] at the European Bioinformatics Institute website (<http://www.ebi.ac.uk/clustalw/>). Phylogenetic analyses were performed using programs from the PHYLIP package [21] and phylogenetic tree was constructed by the neighbor-joining (NJ) algorithm [22] using Kimura ten-parameter distance. The robustness of the inferred tree was evaluated by bootstrap (100 replications).

Antimicrobial Activities Determination

The ability of TN635 strain to produce diffusible metabolites was tested by the agar well diffusion assay [23]. The diameters of the inhibition zones were measured. The bacteriocin samples to be spotted were serially diluted twofold, and the reciprocal of the highest inhibitory dilution was used to calculate the arbitrary activity units (AU) per milliliter. The un-inoculated media were also tested for inhibitory zones as a control.

Effect of Different Treatments on the Antimicrobial Activity of the TN635 Strain

The indicator strains used in these experiments were: *L. ivanovii* BUG 496, *S. enterica* ATCC43972, *S. aureus* ATCC 6538, *E. faecalis* JH 2-2 and *E. faecium* ENSAIA 631, and the pathogenic fungus *C. tropicalis* R2 CIP203. All determinations were carried out simultaneously three times under the same conditions. For each determination, control tests were realized without the corresponding treatment.

Effect of Temperature and Stability at Different pH

Cultures of the TN635 strain in MRS broth under anaerobic condition were maintained at different temperatures (25, 30, 37, and 45°C). The growth was followed by measuring the optical density (OD) at 600 nm. Sensitivity to heat of antimicrobial compounds was investigated by treating the 16-h culture supernatant of the TN635 strain growing at 30°C in water bath at 45, 70, 85, and 100°C for 90 min and 2 h and by autoclaving (20 min at 121°C). In order to determine the sensitivity of the bacteriocin to pH, the supernatant of the TN635 strain culture (30°C, 16 h incubation) was adjusted to pH levels ranging from 1 to 14 (intervals of 1.0) with sterile 1 N HCl or 1 N NaOH and kept at 4°C for 6 h. The treated supernatants were adjusted to pH 7.0 and tested for their antimicrobial activities as previously described.

Sensitivity to Different Enzymes and Surfactants

In order to determine the biological nature of the antimicrobial activity produced by the strain TN635, 1 ml of the cell-free supernatant was incubated for 3 h at 37°C in the presence of 1 mg/ml catalase (Boehringer). The untreated bacteriocin-containing cell-free supernatant served as control. Sensitivity to proteolytic, lipolytic and α -amylase enzymes of antimicrobial compounds was investigated by the addition of Trypsin, pronase E (Sigma),

Proteinase K (Boehringer), lipase A (Sigma) and α -amylase (Sigma) at final concentration of 1 mg/ml to the culture supernatants of the TN635 strain (30°C, 16 h). The samples were incubated for 2 h at 37°C and immediately after, the residual activity was determined as described above. The surfactants used were Tween 20, Tween 80, urea, Triton X-100, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), and phenylmethanesulfonylfluoride (PMSF) which were added to the culture supernatants of the TN635 strain (30°C, 16 h) at a final concentration of 1% (v/v) and incubated at 30°C for 2 h. Surfactants at 1% in MRS broth were used as controls. All surfactants were prepared at 10% in water and filter-sterilized before use.

Effect of Organic Solvents

The culture supernatants of the TN635 strain (30°C, 16 h) were mixed with various organic solvents (ethyl acetate, isopropanol and methanol) at a final concentration of 50% (v/v). After incubation for 1 h at room temperature, the organic solvent was evaporated in a vacuum concentrator and the residual antimicrobial activity was determined.

Bacteriocin Purification, Molecular Weight Determination, and Zymogram Analysis

Strain TN635 was inoculated (1% v/v) into 900 ml (Duran bottles, containing 900 ml of MRS broth), and incubated without agitation at 30°C until early stationary phase (16 h) corresponding to maximum bacteriocin production. For bacteriocin purification, proteins of the crude supernatant fluid (CSF) were precipitated with 40, 60, and 80% saturated ammonium sulfate, gradually added by slow stirring during 4 h at 4°C. After centrifugation at 9,000×g for 30 min at 4°C, the precipitate was suspended in 10 ml of 20 mmol/l sodium phosphate buffer (pH 7.0) and desalted in centrifugal microconcentrators (Amicon, Inc.) with a 10-kDa membrane cutoff. The resulting solution (3.5 ml) was loaded on a column (70×1.5 cm) of gel filtration Sephadex G-25 equilibrated with 20 mmol/l sodium phosphate buffer (pH 7). Elution of proteins was performed with the same buffer at 30 ml/h; 140 fractions (F1–F140) with 5 ml each were eluted from the Sephadex G-25 column and collected automatically. These 140 fractions detected using absorption measurements at 280 nm were fractionated into six samples (S1–S6): S1 (F1–F40), S2 (F41–F70), S3 (F71–F76), S4 (F77–F81), S5 (F82–F93), and S6 (F94–F140). The antimicrobial activity of the different collected fractions was tested using *L. ivanovii* BUG 496, *S. enterica* ATCC43972, and *C. tropicalis* R2 CIP203 as an indicator strains. The active fraction was then applied to a second round of purification by a reverse-phase HPLC method using a C18 column (300×4.6 mm) at a flow rate of 0.5 ml/min. Bacteriocin was eluted from the column with two mobile phases: A (99.9% water, 0.1% trifluoroacetic acid “TFA”) and B (99.9% acetonitrile, 0.1% TFA); from 0 to 5 min (90% A, 10% B), from 5 to 30 min (50% A, 50% B), from 30 to 35 min (20% A, 80% B), from 35 to 50 min (10% A, 90% B) and from 50 to 60 min (90% A, 10% B). Proteins were monitored at 280 nm. The pooled biological active fraction obtained from HPLC elution was concentrated and stored at –20°C. The estimated molecular weight of the purified bacteriocin was determined by SDS-PAGE according to Laemmli [24] with 20% acrylamide gel. Protein concentration was measured using BSA as reference as described by Bradford [25]. The used protein marker is from BioLabs with a broad range of 2–212 kDa. To determine the apparent molecular mass of the bacteriocin, the gel was cut into two vertical parts after SDS-PAGE. The part of the gel containing the sample and the protein marker was stained with 0.25% Coomassie brilliant blue R-250 (BioRad), while the remaining part, containing only the sample, was washed in 10 mmol/l phosphate buffer (pH 7) for 4 h, then

fixed and used for direct detection of antimicrobial activity by overlaying with LB soft agar containing 100 µl of a 5 h culture of *L. ivanovii* BUG 496.

Mode of Action of BacTN635

For this realization, we have used two indicator microorganisms: *L. ivanovii* BUG 496 and *C. tropicalis* R2 CIP203. Partially-purified BacTN635 (the fraction obtained from gel filtration chromatography and freeze-dried) at 400 AU/ml, was added to 200 ml LB culture of *L. ivanovii* BUG 496 and 200 ml YP10 of *C. tropicalis* R2 CIP203 at in early exponential phase (10^7 colony-forming units (CFU)/ml). The two indicator microorganisms growing in LB (*L. ivanovii* BUG 496) and YP10 (*C. tropicalis* R2 CIP203) in the absence of bacteriocin were used as controls. Changes in the turbidity of the cultures were recorded at an O.D. of the 600 nm and the number of CFU was determined by plating the samples on LB or YP10 agar.

Results and Discussion

Screening of LAB Strains Producing Bacteriocin

During previous works (data not shown), a collection of 54 LAB strains were isolated from several isolates (meat, fermented vegetables, and dairy products). We have tested these bacteria for their capacity to produce antimicrobial activities against the following indicator microorganisms: *Lb. casei* DSM 20011, *L. ivanovii* BUG 496, *S. enterica* ATCC43972, *Fusarium* sp., and *C. tropicalis* R2 CIP203. As shown in Table 1, different profiles of inhibition were observed. Nine strains (TN600, TN606, TN615, TN618, TN623, TN627, TN635, TN644, and TN653) were characterized by the broad-spectrum inhibition. These nine isolates were tested for their capacity to inhibit the growth of other microorganisms (Table 2). The largest spectrum of inhibition was showed by TN635 strain which inhibited all used indicator microorganisms. Moreover, the active molecule(s) of the TN635 strain presents the highest inhibitory effect against all tested microorganisms including the pathogenic Gram-positive bacteria (*L. ivanovii* BUG 496, *S. aureus* ATCC 6538, *E. faecalis* JH 2-2 and *E. faecium* ENSAIA 631), the four pathogenic Gram-negative bacteria (*S. enterica* ATCC43972, *P. aeruginosa* ATCC 49189, *Hafnia* sp. and *Serratia* sp.), and the pathogenic fungus *C. tropicalis* R2 CIP203. Since bacteriocins often have a narrow killing spectrum and inhibit only bacteria closely related to those from which they have been formed, bacteriocins from Gram-positive bacteria are generally not effective against Gram-negative bacteria and have no effect with yeasts and fungi [26, 27]. The search for new bacteriocins with a wider spectrum of activity which can be used in human health, agriculture, and the food industry is being studied by several research groups. According to its large inhibitory spectrum, our finding, BacTN635 of the new isolated TN635 strain, may be of applied interest as food preservation and/or in the protection of human health.

Identification and Phylogenetic Analysis of TN635 Strain

Using the two specific primers: forward P1: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse P2: 5'-ATGGCTCAGGACGAACGCT-3' and the TN635 strain genomic DNA as template, we have amplified by PCR a DNA fragment having an expected size of approximately 1,500 pb. After purification, this DNA fragment was cloned in the pGEM-T

Table 1 Inhibitory spectrum (zone of inhibition/mm) of the isolated 54 LAB (TN600- TN653) against *Lb. casei* DSM 20011, *L. ivanovii* BUG 496, *S. enterica* ATCC43972, *Fusarium*.sp, and *C. tropicalis* R2 CIP203; *L* (*listeria*); *Lb* (*Lactobacillus*); *C* (*Candida*).

Isolates	Indicator microorganisms				
	<i>Lb. casei</i> DSM 20011	<i>L. ivanovii</i> BUG 496	<i>S. enterica</i> ATCC43972	<i>Fusarium sp.</i>	<i>C. tropicalis</i> R2 CIP203
TN600	6	12	22	14	8
TN601	–	7	–	–	–
TN602	–	–	–	–	–
TN603	8	–	–	–	6
TN604	–	–	–	8	8
TN605	10	16	7	7	–
TN606	7	18	16	16	9
TN607	6	5	–	–	–
TN608	8	8	8	–	–
TN609	9	–	7	8	–
TN610	–	13	15	–	–
TN611	–	–	–	–	–
TN612	–	–	–	–	–
TN613	9	17	10	–	8
TN614	–	6	7	8	15
TN615	7	6	18	19	5
TN616	8	–	–	16	–
TN617	–	–	–	18	–
TN618	8	14	16	18	9
TN619	8	7	–	10	–
TN620	7	16	–	–	–
TN621	6	–	–	20	–
TN622	–	7	5	–	6
TN623	7	20	18	17	19
TN624	8	8	7	7	–
TN625	7	6	17	–	–
TN626	–	–	–	–	–
TN627	8	9	12	14	10
TN628	–	–	6	16	–
TN629	7	–	–	6	–
TN630	9	9	–	6	6
TN631	–	18	17	9	–
TN632	–	8	8	–	–
TN633	9	–	–	–	7
TN634	6	–	7	7	–
TN635	9	22	21	23	21
TN636	9	6	–	7	–
TN637	8	7	–	15	–
TN638	7	–	7	16	–
TN639	–	7	7	18	7

Table 1 (continued).

Isolates	Indicator microorganisms				
	<i>Lb. casei</i> DSM 20011	<i>L. ivanovii</i> BUG 496	<i>S. enterica</i> ATCC43972	<i>Fusarium sp.</i>	<i>C. tropicalis</i> R2 CIP203
TN640	7	7	8	6	–
TN641	–	16	17	–	15
TN642	6	–	–	16	–
TN643	–	6	7	17	6
TN644	7	16	6	22	6
TN645	6	7	8	–	–
TN646	–	–	–	18	7
TN647	7	–	6	8	–
TN648	8	8	–	18	–
TN649	–	–	8	16	6
TN650	–	16	–	17	17
TN651	6	–	7	7	6
TN652	–	8	–	12	7
TN653	7	16	17	18	8

Table 2 Inhibitory spectrum (zone of inhibition/mm) of TN600, TN606, TN615, TN618, TN623, TN627, TN635, TN 644 and TN653 strains against Gram-positive and Gram-negative bacteria, LAB and fungi: *L* (*listeria*); *Lb* (*Lactobacillus*); *Lc* (*Lactococcus*); *C* (*Candida*).

Indicator strains	TN600	TN606	TN615	TN618	TN623	TN627	TN635	TN644	TN653
Gram-positive bacteria									
<i>M. luteus</i> LB 14110	23	14	6	14	14	6	23	14	11
<i>S. aureus</i> ATCC 6538	12	15	16	14	9	16	22	21	8
<i>L. ivanovii</i> BUG 496	12	18	6	16	20	9	22	16	16
<i>E. faecalis</i> JH 2-2	6	18	8	–	15	16	22	7	8
<i>E. faecium</i> ENSAIA 631	6	14	7	–	14	14	21	8	6
Gram-negative bacteria									
<i>E. coli</i> ATCC 8739	5	22	13	15	15	23	22	13	22
<i>S. enterica</i> ATCC43972	22	16	18	16	18	12	21	6	17
<i>P. aeruginosa</i> ATCC 49189	22	16	6	9	14	9	22	8	9
<i>Hafnia sp.</i>	5	15	6	13	24	6	24	5	7
<i>Serratia sp.</i>	12	6	12	15	21	7	23	8	9
LAB									
<i>Lb. Casei</i> DSM 20011	6	7	7	8	7	8	9	7	7
<i>Lb. delbrueckii</i> DSM 20081	–	6	8	–	–	9	9	6	–
<i>Lb. Sakei</i> 2525	–	6	7	–	7	6	9	–	–
<i>Lc. lactis</i> ATCC 11454	–	–	6	5	6	7	9	–	9
<i>Lc. lactis cremoris</i> DSM 11603	–	–	5	7	7	9	8	–	8
Fungi									
<i>Fusarium sp.</i>	14	16	19	18	17	14	23	22	18
<i>C. tropicalis</i> R2 CIP203	8	9	5	9	19	10	21	6	8

Easy vector, yielding the pSS1 plasmid. Total nucleotide sequence of 1,540 pb (accession number FN252881) of the whole 16S rRNA gene of strain TN635 was determined in both strands. The alignment of this sequence through matching with reported 16S rRNA gene sequences in gene bank shows high similarity (97–99%) to the *Lactobacillus* 16S rRNA genes. The organism most similar to the new isolate TN635 strain was *L. plantarum* NRIC 0383 (Fig. 1). Based on the nucleotide sequence of the 16S rRNA gene of the TN635 strain and the phylogenetic analysis, we propose the assignment of our new isolate bacterium as *L. plantarum* sp. TN635 strain.

Growth and Bacteriocin Production

To test the effect of incubation temperature on cell growth and antimicrobial activity production, cultures of the *L. plantarum* sp. TN635 strain in MRS broth under anaerobic conditions were maintained at different temperatures (25, 30, 37, and 45°C). The growth was followed by measuring the OD at 600 nm and the antimicrobial activity was determined against the three indicator microorganisms: *L. ivanovii* BUG 496, *S. enterica* ATCC43972, and *C. tropicalis* R2 CIP203. Aliquots were withdrawn at 3 h time intervals. Obtained results show that the secretion of biological activity is correlated with biomass production. Highest growth and antimicrobial activity were obtained at 30°C (Fig. 2). We can deduce that the extracellularly antimicrobial activity was produced in the logarithmic growth phase and the production profile was a typical growth associated pattern. The maximum antimicrobial activity was detected 16 h after incubation. This activity remains stable between 13 and 20 h and then gradually decreased.

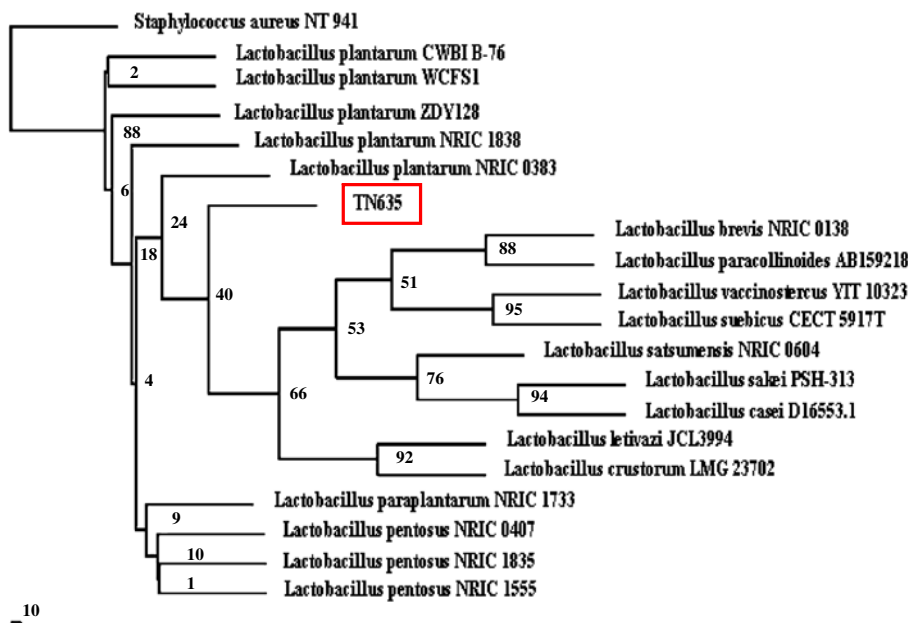


Fig. 1 Phylogenetic trees derived from 16S rDNA sequence of *L. plantarum* sp. TN635 strain. All the sequences used here were from LAB type strains

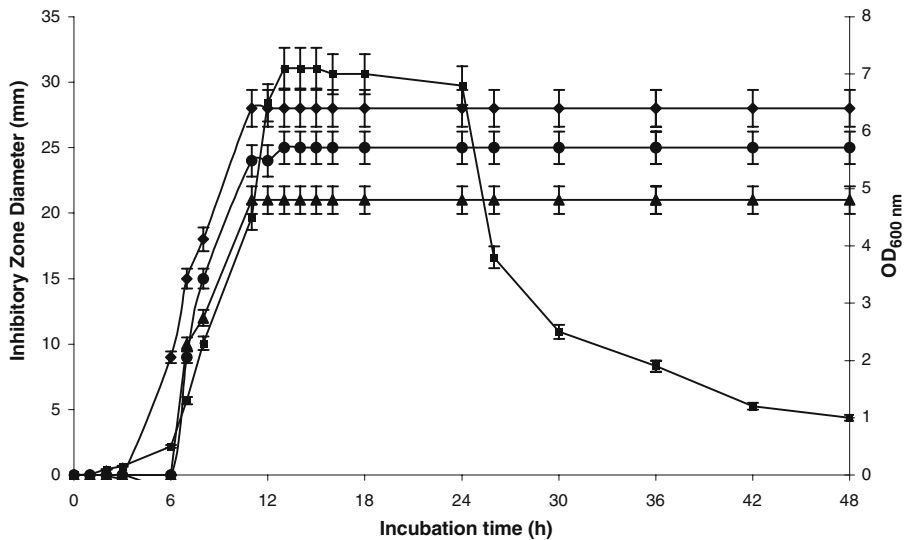


Fig. 2 BacTN635 production during growth of *L. plantarum* TN635 in MRS broth at 30°C. (filled square) Optical density at 600 nm; activities of BacTN635 against *C. tropicalis* R2 CIP203 (filled upright triangle); against *S. enterica* ATCC43972 (filled circle), and against *L. ivanovii* BUG 496 (filled diamond) were measured by diameter (mm) of zone of inhibition

Effects of Enzymes, Heat, pH, Surfactants and Organic Solvents

Enzymatic tests showed that the antimicrobial activity against the three tested indicator microorganisms, *L. ivanovii* BUG 496, *S. enterica* ATCC43972, and *C. tropicalis* R2 CIP203, of the supernatant culture of the *L. plantarum* sp. TN635 strain was not affected by the addition of catalase, indicating that the observed growth inhibition was not due to hydrogen peroxide production. In contrast, treatment with the proteolytic enzymes (Trypsin, pronase E, and Proteinase K) caused complete inactivation of the antimicrobial compounds, which therefore identified them as proteinaceous substances. The sensitivity of the inhibitory compounds to heat treatment showed that inhibitory activity was not significantly altered by boiling for 90 min showing that the antagonistic activity of the culture supernatant is heat-resistant. The activity of the sample was insensitive to lipase and α -amylase, which eliminates the possibility that synthesized active compounds are included in the group of complex biologically active substances containing a lipid or carbohydrate component [28]. Concerning the sensitivity to pH, the supernatant culture of the *Lb. plantarum* sp. TN635 (30°C, 16 h incubation) was adjusted to pH levels ranging from 1 to 14 (intervals of 1.0). The obtained results show that bacteriocin activity was highest when the pH of the supernatant was between 3 and 11, with an optimum at pH 7. About 50% activity was maintained at pH 12 and was completely inactivated above pH 12. Exposure to surfactants caused about 15% inactivation of the antimicrobial activity for the SDS agent and about 40% for the other tested surfactants (Tween 20, Tween 80, Triton X-100, Urea, EDTA, and PMSF). Concerning the treatment with organic solvents (ethyl acetate, methanol, and isopropanol), no significant alteration of the BacTN635 activity was observed.

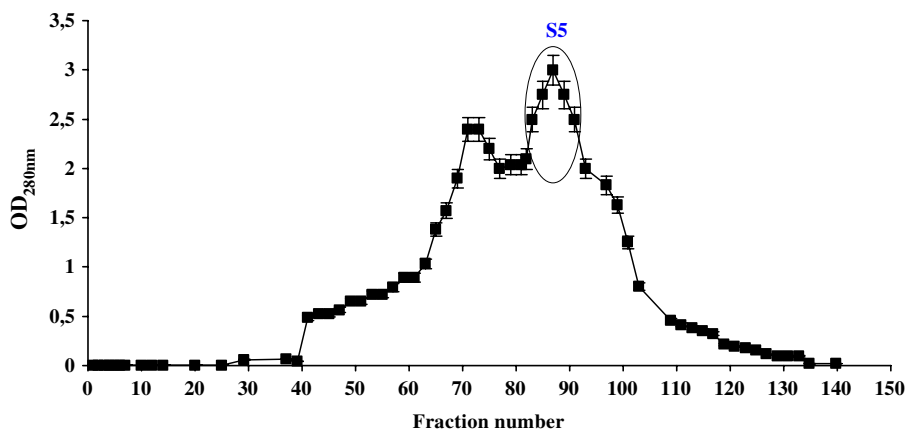


Fig. 3 Purification of BacTN635 by cation-exchange chromatography using Sephadex G-25 monitoring by absorbance at 280 nm (filled square)

Bacteriocin Purification and Molecular Weight

BacTN635 was purified to homogeneity from a cell-free culture supernatant of *Lb. plantarum* sp. TN635 strain by a four-step protocol. Maximum antimicrobial activity was obtained when the cell-free culture supernatant was precipitated with 80% ammonium sulfate. One hundred forty fractions (F1–F140) with 5 ml each were eluted from a Sephadex G-25 column. These 140 fractions detected using absorption measurements at 280 nm were fractioned into six samples (S1–S6). After concentration, the antagonistic activity of each sample was assayed using *L. ivanovii* BUG 496, *S. enterica* ATCC43972, and *C. tropicalis* R2 CIP203 as indicator microorganisms. Only sample S5 (F82–F93) possesses inhibitory activity against the three tested cells (Fig. 3). Sample S5 was then applied to another round of purification using the HPLC technique. Three well-defined

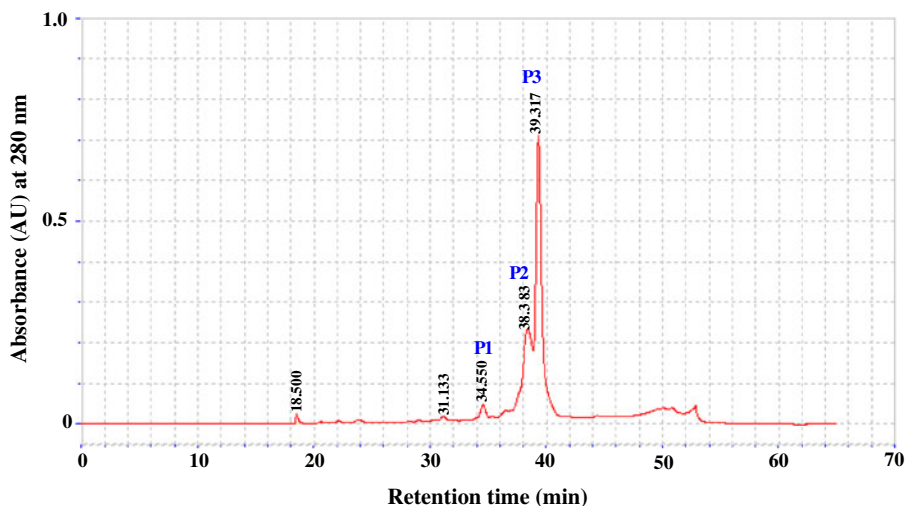


Fig. 4 Elution profile of bacteriocin using HPLC reverse-phase chromatography on C18 column monitoring by absorbance at 280 nm

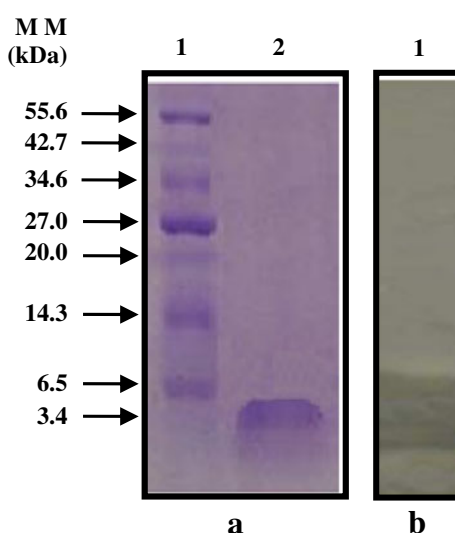
Table 3 Purification of BacTN635 produced by *Lactobacillus plantarum* sp. TN635 strain.

Step	Volume (ml)	Total activity (AU) ^a	Total protein (mg)	Specific activity (AU/mg)	Purification fold	Yield %
Culture supernatant	900	45×10^5	2,160	2,083	1	100
Ammonium sulfate precipitation	50	5.2×10^5	52.5	9,904	4.75	11.55
Microconcentrators	32	3.32×10^5	23.2	14,310	6.87	7.38
Gel filtration	3.5	2.25×10^5	1.54	146,104	70.14	5
HPLC	0.7	1.125×10^5	0.57	197,368	94.75	2.5

^a Antibacterial activity (in arbitrary units [AU]) was assayed by agar well diffusion assay using *L. ivanovii* BUG 496 as an indicator strain

peaks (P1–P3) were obtained having, respectively, the following retention times: P1: 34.55 min, P2: 38.38 min, and P3: 39.31 min (Fig. 4). Only the fraction of the peak P3 possesses an antimicrobial activity against the three tested microorganisms mentioned above and no activity has been observed for the two other fractions. The yield, activity, and purification fold of the BacTN635 of the four various purification steps were summarized in Table 3. SDS-PAGE analysis of the purified BacTN635, revealed a single band with an estimated molecular mass of approximately 4 kDa indicating that this bacteriocin had been purified to homogeneity (Fig. 5a). Antibacterial activity determination against *L. ivanovii* BUG 496 strain, reveals a growth inhibitory zone at the same position than that visualized in the stained gel (Fig. 5b). During the last few years, a large number of new bacteriocins produced by LAB bacteria have been identified and characterized. However, a few bacteriocins produced by this species have been reported to be active against Gram-negative bacteria and particularly against fungi. Most of the described bacteriocins as antifungal agents from *Lactobacillus* are organic compounds or diketopiperazine derivatives with low molecular mass, lower than 1 kDa [29]. Too rare are the bacteriocins described from LAB bacteria with a molecular mass higher than 1 kDa and having antifungal activities [30].

Fig. 5 Electrophoretic and zymogram analyses of the purified BacTN635. Coomassie brilliant blue R-250 stained SDS-PAGE gel. Lane 1 molecular mass markers; lane 2 purified BacTN635 (a). Portion of the renaturated SDS-PAGE, overlaid with LB soft agar containing *L. ivanovii* BUG 496 (b)



Mode of Action of the BacTN635

The addition of partial purified BacTN635 (the fraction obtained from gel filtration chromatography and freeze-dried) at 400 AU/ml, to cells of *L. ivanovii* BUG 496 (3 h old) and *C. tropicalis* R2 CIP203 (10 h old) in their early logarithmic growth phase, resulted in a rapid decrease in the number of *L. ivanovii* BUG 496 viable cells (from 10^7 CFU/ml to less than 10 CFU/ml) over a period of 5 h (Fig. 6a). The optical density readings of this indicator microorganism remained constant since the addition of the BacTN635 (Fig. 6a). These results indicate that the studied bacteriocin exhibits bactericidal effect against *L. ivanovii* BUG 496 strain. Concerning the *C. tropicalis* R2 CIP203, the optical density readings were very similar in presence and absence of BacTN635 (Fig. 6b). However, we notice an increase of the number of viable cells grown in the absence of the bacteriocin at

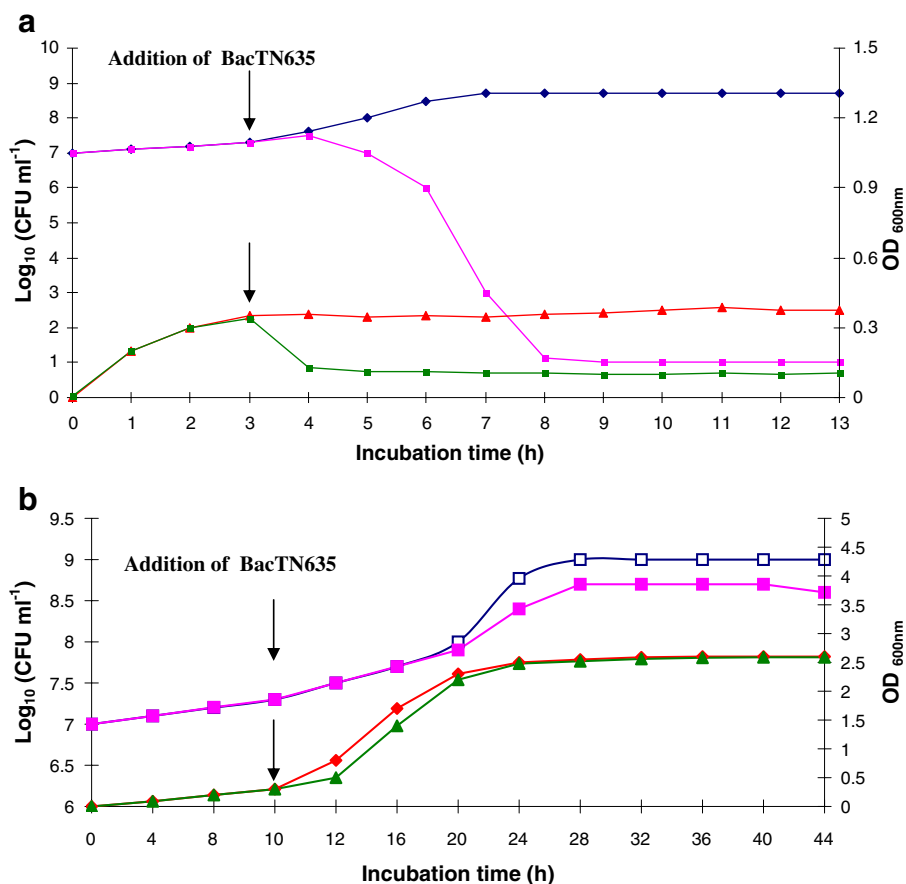


Fig. 6 The effect of BacTN635 on the growth of *L. ivanovii* BUG 496. Optical density at 600 nm in absence (▲) and in presence (▴) of BacTN635. Viable cell counts (CFU/ml) in absence (♦) and in presence (■) of BacTN635 (a). The effect of BacTN635 on the growth of *C. tropicalis* R2 CIP203. Optical density at 600 nm in absence (♦) and in presence (▲) of BacTN635. Viable cell counts (CFU/ml) in absence (□) and in presence (■) of BacTN635 (b)

40 h of growth (Fig. 6b). These data indicate that BacTN635 acts with a fungistatic effect against *C. tropicalis* R2 CIP203.

Conclusions

A new LAB strain has been selected for its antimicrobial activity against pathogenic Gram-negative bacteria and fungi. Based on the analysis of the nucleotide sequence (1,540 pb) of the whole 16S rRNA gene (accession no: FN252881) and the phylogenetic study, we propose the assignment of our new isolate bacterium as *Lb. plantarum* sp. TN635 strain. The antimicrobial compound of this strain was determined as a proteinaceous substance. The maximum bacteriocin-like substance (BacTN635) production (5,000 AU/ml) was recorded after a 16-h incubation in MRS medium at 30°C. BacTN635 was stable to heat and to treatment with surfactants and organic solvents. Highest antimicrobial activity was found between pH 3 and 11 with an optimum at pH=7. The purified BacTN635, revealed a single band with an estimated molecular mass of approximately 4 kDa. BacTN635 produced by *Lb. plantarum* sp. TN635 strain, may possess potential practical applications, since it was able to inhibit important Gram-negative pathogenic bacteria such as (*S. enterica* ATCC43972, *P. aeruginosa* ATCC 49189, *Hafnia* sp., and *Serratia* sp.) and pathogenic fungi such as *C. tropicalis* R2 CIP203. *S. enterica* is an important cause of human salmonellosis and food-poisoning associated with consumption of contaminated chicken eggs and poultry products [31]. *P. aeruginosa* is an opportunistic pathogen of humans. It causes urinary tract infections, respiratory system infections, gastrointestinal infections, and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed [32]. *Serratia* and *Hafnia* sp. are usually found in the intestinal tract. These pathogenic bacteria cause a wide variety of infections primarily pneumonia, wound, and urinary tract infections. Moreover, it should be noted that the number of patients with fungal infections has been rising in recent years. In addition to the species *Candida albicans* and *Candida stellatoidea*, *C. tropicalis* is one of the significant pathogenic yeasts in humans belonging to the genus *Candida* [33].

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References

1. Alisky, J., Iczkowski, K., Rapoport, A., & Troitsky, N. (1998). *Journal of Infection*, 36, 5–15.
2. Macfarlane, G. T., & Cummings, J. H. (2002). *Current Opinion in Infectious Diseases*, 15, 501–506.
3. Papagianni, M. (2003). *Biotechnology Advances*, 21, 465–499.
4. Twomey, D., Ross, R. P., Ryan, M., Meaney, B., & Hill, C. (2002). *Antonie Van Leeuwenhoek*, 82, 165–18.
5. Brooks, G. F., Butel, J. S., & Morse, S. A. (1998). *Jawetz, Melnick, and Adelberg's medical microbiology* (p. 269). Upper Saddle River, New Jersey, USA: Appleton & Lange.
6. Settanni, L., & Corsetti, A. (2008). *International Journal of Food Microbiology*, 121, 123–138.
7. Nawaz, S. K., Riaz, S., Riaz, S., & Hasnain, S. (2009). *African. J. Biotechnol.*, 8, 365–368.
8. Hammami, I., Rhouma, A., Jaouadi, B., Rebai, A., & Nesme, X. (2009). *Letters in Applied Microbiology*, 48, 253–260.

9. Diop, M. B., Dubois-Dauphin, R., Tine, E., Ngom, A., Destain, J., & Thonart, P. (2007). *Biotechnology, Agronomy, Society and Environment*, 11, 275–281.
10. Paul, R. R., Morgan, S., & Hill, C. (2002). *International Journal of Food Microbiology*, 79, 3–16.
11. Messi, P., Bondi, M., Sabia, C., Battini, R., & Manicardi, G. (2001). *International Journal of Food Microbiology*, 64, 193–198.
12. Todorov, S. D., & Dicks, L. M. (2004). *Journal of Basic Microbiology*, 44, 305–316.
13. Todorov, S. D., & Dicks, L. M. (2005). *Enzyme and Microbial Technology*, 36, 318–326.
14. Todorov, S. D., Nyati, H., Meincken, M., & Dicks, L. M. T. (2007). *Food Control*, 18, 656–664.
15. Hanahan, D. (1983). *Journal of Molecular Biology*, 16, 557–580.
16. Janssen, G. R., & Bibb, M. J. (1993). *Gene*, 124, 133–134.
17. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (2nd ed.). New York: Cold Spring Harbor Laboratory Press.
18. Leenhouts, K. J., Kok, J., & Venema, G. (1990). *Applied and Environmental Microbiology*, 56, 2726–2735.
19. Lee, C. M., Siew, C. C., Abdullah, N., & Ho, Y. W. (2008). *FEMS Microbiology Letters*, 287, 136–141.
20. Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). *Nucleic Acids Research*, 22, 4673–4680.
21. Felsenstein, J. (1989). PHYLIP-Phylogeny Inference Package, version 3.2. *Cladistics*, 5, 164–166.
22. Saitou, N., & Nei, M. (1987). *Molecular Biology and Evolution*, 4, 406–425.
23. Tagg, J. R., & McGiven, A. R. (1971). *Applied Microbiology*, 21, 943.
24. Laemmli, U. K. (1970). *Nature*, 227, 680–685.
25. Bradford, M. M. (1976). *Analytical Biochemistry*, 72, 248–254.
26. Karunasagar, I., Krohne, G., & Goebel, W. (1993). *Infection and Immunity*, 61, 162–169.
27. Atanassova, V., Meindl, A., & Ring, C. (2001). *International Journal of Food Microbiology*, 68, 105–113.
28. Aktypis, A., Kalantzopoulos, G., Huis IN'T Veld, J. H. J., & Ten Brink, B. (1998). *Journal of Applied Microbiology*, 84, 568–576.
29. Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., & Gobetti, M. (2000). *Applied and Environmental Microbiology*, 66, 4084–4090.
30. Atanassova, M., Choiset, Y., Dalgalarond, D., Chobert, J. M., Dousset, X., Ivanova, I., et al. (2003). *International Journal of Food Microbiology*, 8, 63–73.
31. Erich, L., & Martin, J. (1997). Antimicrobial food additives, characteristics, uses, effects (2nd Ed.). Translated by S.F. Laichena Berlin: Springer.
32. Wiblin, R. T. (1997). Nosocomial pneumonia. In R. P. Wenzel (Ed.), *Prevention and control of nosocomial infections* (3rd ed., pp. 807–819). Baltimore: Williams and Wilkins.
33. Kobayashi, H., Matsuda, K., Ikeda, T., Suzuki, M., Takahashi, S., Suzuki, A., et al. (1994). *Infection and Immunity*, 62, 615–622.