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Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF

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Abstract

Lactobacillus plantarum ST8KF, isolated from kefir, produced a 3.5 kDa bacteriocin (bacST8KF) active against *Lb. casei*, *Lb. salivarius*, *Lb. curvatus* and *Listeria innocua*. BacST8KF was sensitive to proteolytic enzymes, but stable between pH 2.0 and 10.0, and heat resistant (20 min at 121 °C). BacST8KF did not adsorb to the surface of the producer cell. Maximum activity (25,600 AU mL^{-1}) was recorded in MRS broth with glucose, in MRS broth with glucose replaced by sucrose, and in MRS broth with glucose, supplemented with KH_2PO_4 after 24 h at 30 °C. Tri-ammonium citrate and glycerol in excess of 5.0 g L^{-1} repressed bacST8KF production. Production of bacST8KF increased from 800 AU mL^{-1} after 3 h of fermentation in MRS broth at 30 °C to 12,800 AU mL^{-1} after 9 h and to 51,200 AU mL^{-1} after 27 h. These results suggest that bacST8KF may be a secondary metabolite and shows that its mode of activity is bacteriostatic.

Keywords: Bacteriocin ST8KF; *Lactobacillus plantarum*; Kefir

1. Introduction

Kefir is a refreshing, naturally carbonated fermented milk beverage with a slightly acidic taste (Gulmez & Guven, 2003; Tamine & Marshall, 1997), yeasty flavour and creamy consistency (Duitschaever, 1989; Wickerham, 1951). When agitated, the beverage foams and fizzes (Duitschaever, 1989; Obermann, 1985), a characteristic that led to kefir being named "the champagne of cultured dairy products" (Merin & Rosenthal, 1986).

Fermentation is initiated by the addition of kefir grains to fresh milk (Garrote, Abraham, & DeAntoni, 2000). The grains are insoluble in water and irregular in shape and size, varying from 0.3–3.5 cm in diameter (Garrote, Abraham, & DeAntoni, 1997; Kosikowski & Mistry, 1997). When suspended in milk, the grains swell to form a gelatinous-like product, kefiran (Kosikowski, & Mistry, 1997), which contains lactic acid bacteria (LAB), yeast and acetic acid bacteria (Saloff-Coste, 1996). The majority of bacteria (as much as 80%) belong to the genus *Lactobacillus* (Witthuhn, Schoeman, & Britz, 2004). The microbial composition of kefir is determined by the source of the grains (Ottogalli, Galli, Resmini, & Volonterio, 1973; Witthuhn et al., 2004), the fermentation process (Molska, Moniuszco, Komorows, & Merilanen, 1983) and storage conditions (Zourari & Anfantakis, 1988). Filamentous fungi have been described, but are only present in low numbers (Garrote et al., 1997; Saloff-Coste, 1996).

Many health benefits have been attributed to kefir, including its antimicrobial activity against a range of Gram-positive and Gram-negative bacteria, and fungi (Garrote et al., 2000; Saloff-Coste, 1996). In in vitro tests with cell-free extracts of kefir, the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Clostridium tyrobutyricum* and *Listeria monocytogenes* was inhibited (Van Wyk, 2001). In general, the antimicrobial activity of kefir is ascribed to lactic acid, volatile acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, and/or bacteriocins produced by LAB (Havenaar, Brink, & Sanders, 1993; Helander, Von Wright, Huis in't Veld, 1992; Mattila-Sandholm, 1997).

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In an in vitro study conducted by Rodrigues, Caputo, Carvalho, Evangelista, and Schneedorf (2005), kefir inhibited the growth of *Streptococcus pyogenes* and *Candida albicans*. In another study, strains of *Lactococcus cremoris*, *Lc. lactis*, *Str. thermophilus* and *Str. durans*, isolated from kefir inhibited the growth of *S. aureus* (Yüksekdag, Beyatli, & Aslim, 2004). In the same study, two strains of *Lc. lactis* and a strain of *Lc. cremoris* inhibited the growth of *E. coli* and *Pseudomonas aeruginosa* (Yüksekdag et al., 2004). These authors also described a strain of *Str. thermophilus* active against *P. aeruginosa*. Atanassova, Dousset, Montcheva, Ivanova, and Haertle (1999) described a bacteriocin produced by a strain classified as *Lactobacillus* spp. with activity against *L. innocua* F. A number of *Lactobacillus* spp. isolated from kefir displayed antimicrobial activity against enteropathogenic bacteria and affected the adhesion of *Salmonella typhimurium* to Caco-2 cells (Santos, San Mauro, Sanchez, Torres, & Marquina, 2003).

The objective of this study was to characterize bacteriocin ST8KF, produced by *Lb. plantarum* strain ST8KF isolated from kefir, with the aim of using the strain as a co-starter culture in kefir fermentations.

2. Materials and methods

2.1. Isolation of lactic acid bacteria and screening for bacteriocin activity

A combination of kefir (50 mL) and kefir grains (5 g), obtained from the Department of Food Science, Stellenbosch University, was macerated in a stomacher (BagMixer, Interscience, Weymouth, USA) for 10 min at 25 °C. Serial dilutions of the sample were made with sterile saline (0.85%, w/v NaCl), plated onto MRS agar (Biolab Diagnostics, Midrand, SA) and incubated at 30 °C for 24 h.

Screening for bacteriocin-producing isolates was carried out according to the triple-agar-layer method described by Todorov and Dicks (2005b). The second layer of agar (1.7%, w/v) was supplemented with 50.0 mg L^{-1} Natamycin (Delvocid®, Gist-brocades, B.V., Delft, The Netherlands) to prevent fungal growth. All plates were incubated at 30 °C for 24 h. Colonies were then overlaid with a third layer of 1% (w/v) brain heart infusion (BHI) agar (Biolab), seeded with 10^6 cfu mL^{-1} *Lb. casei* LHS, *E. coli* ATCC 11775 and *S. aureus* ATCC 12600, respectively. The plates were incubated at 37 °C for 24 h. Colonies with the largest zones of growth inhibition were isolated, inoculated into MRS broth (Biolab) and incubated for 24 h at 30 °C. Pure cultures were obtained by streaking onto MRS agar (Biolab).

Antimicrobial activity was confirmed by using the agar-spot-test method (Van Reenen, Dicks, & Chikindas, 1998). Activity was expressed as arbitrary units (AU) per mL, with one AU defined as the highest dilution showing a clear zone of inhibition (Todorov & Dicks, 2005b; Van Reenen et al., 1998). *Lb. casei* LHS was used as a sensitive test strain.

2.2. Identification of strain ST8KF

The morphology of strain ST8KF was determined by using a scanning electron microscope (Leo® 1430VP, Carl Zeiss Jena GmbH, Jena, Germany). Cells cultured for 24 h in MRS broth (Biolab) were harvested (8 000 × *g*, 10 min, 4 °C), washed with sterile distilled water, and resuspended in 1 mL sterile distilled water. Prior to imaging, samples were sputter-coated with either gold or carbon, depending on the application. Samples were identified with back-scattered electron (BSE) and/or secondary electron images, and phase compositions quantified by energy dispersive spectrometer (EDS) analysis using an Oxford Instruments, location 133KeV detector and Oxford INCA software (Oxford Instruments, Witney, Oxon, UK). Beam conditions during the quantitative analyses were 20 KV and approximately 1.5 nA, with a working distance of 13 mm and a specimen beam current of −3.92 nA. Despite the relatively low energy of the beam, X-ray counts with the set-up used were typically ~5000 counts per second (cps). The counting time was 50 s live-time. Natural mineral standards of Co, Ti and Fe were used for standardization and verification of the analyses. Pure Co, as well as Ti and Fe in ilmenite were used periodically to correct for detector drift. Beam conditions during semi-quantitative analyses, when used in case of unpolished samples, were as described above without controlling the specimen beam current and the results were normalised to 100% weight.

Further identification was by gram-reaction, and physiological and biochemical tests, as described by Schillinger and Lücke (1987), Stiles and Holzapfel (1997) and Collins, Phillips, and Zanoni (1989). Carbohydrate fermentation reactions were recorded by using the API 50 CHL test kit (BioMérieux S.A., Marcy I'Etiole, France).

Identification to species level was by PCR with primers specific for *Lb. plantarum* (PlanF and REV), *Lb. pentosus* (PentF and REV), and *Lb. paraplantarum* (ParaF and REV), as described by Torriani, Felis, and Dellaglio (2001). Confirmation of identification was obtained by amplifying the genomic DNA with primers F8 and R1512, as described by Felske, Rheims, Wolterink, Stackebrandt, and Akkermans (1997). The amplified fragments were cleaned using SigmaSpin™ Post-Reaction Clean-Up Columns (Sigma, St Louis, MO, USA), sequenced, and compared to sequences in GenBank using BLAST, Basic Local Alignment Search Tool (Altschul, et al., 1997).

2.3. Isolation of bacteriocin ST8KF

Strain ST8KF was cultured in MRS broth (Biolab) for 24 h at 30 °C. The cells were harvested (8000 × *g*, 10 min, 4 °C), the cell-free supernatant was adjusted to pH 5.0 with 1 M NaOH, heat-treated (80 °C for 10 min) and the bacteriocin (bacST8KF) precipitated with 80% saturated

ammonium sulphate (Sambrook, Fritsch, & Maniatis, 1989). The precipitate was resuspended in 20 mL of 25 mM ammonium-acetate (pH 6.5) and the amount of antimicrobial activity determined by testing against *Lb. casei* LHS, as described above.

The molecular size of bacST8KF was determined by tricine/SDS–PAGE (Schägger & Von Jagow, 1987). A low molecular weight marker with sizes ranging from 2.5 to 45.0 kDa (Amersham Bioscience Europe GmbH, Freiburg, Germany) was used. One half of the gel was covered with *Lb. casei* LHS (10^6 cfu mL^{-1}) imbedded in 1% (w/v) BHI agar (Biolab) and incubated at 37 °C for 24 h. The other half was stained with Coomassie Brilliant Blue R (ICN Biomedicals Inc., 1263 South Chillicothe Rd, Aurora, Ohio 44202, USA).

2.4. Effect of enzymes, pH, detergents and temperature on bacST8KF

One millilitre of a cell-free supernatant, prepared as described before, was added to 1 mg mL^{-1} α-amylase (Sigma Diagnostics, St. Louis, MO, USA), 1 mg mL^{-1} Proteinase K (Roche, Indianopolis, IN, USA) and 1 mg mL^{-1} pronase (Roche), respectively. Samples were incubated at 30 °C for 30 min and then heated at 95–97 °C for 5 min. In a separate experiment the pH of 10 mL of cell-free supernatants was adjusted to 2.0, 4.0, 6.0, 8.0 or 10.0 with 1 M HCl or 1 M NaOH and incubated at 30 °C for 1 h. Another batch of cell-free supernatants received 10 mg mL^{-1} of Triton X100 (BDH Chemicals Ltd, Poole, England), Triton X114 (Sigma), Tween 20 (Merck, Darmstadt, Germany), Tween 80 (Merck), SDS (Sigma), urea (Merck) or EDTA (Merck), respectively, and incubated for 30 min at 30 °C. The effect of temperature on bacST8KF was determined by incubating cell-free supernatants at 30, 45, 60 and 100 °C for 30 min and 2 h, respectively, and at 121 °C for 20 min. The pH of all samples was adjusted to 5.0 and bacST8KF activity determined with *Lb. casei* LHS as sensitive strain, as described above.

2.5. Production of bacST8KF

Two millilitre of a 24 h culture was inoculated into 100 mL MRS broth (Biolab) and incubated at 30 °C. Changes in optical density (600 nm) and pH were determined hourly for 30 h. BacST8KF activity was determined every three hours, as described above.

2.6. Mode of bacST8KF activity

MRS broth (Biolab) was inoculated with 1% (v/v) *Lb. casei* LHS and incubated for 3 h at 37 °C. Twenty millilitres filter-sterilized cell-free supernatant was added to the culture and changes in optical density (at 600 nm) recorded every hour for 12 h.

2.7. Adsorption of bacST8KF to producer cells

Adsorption of bacST8KF to producer cells was studied by the method of Yang, Johnson, and Ray (1992). An 18 h-old culture was adjusted to pH 5.0 with 1 M NaOH, 10 mL of the cells harvested ($8000 \times g$, 15 min, 4 °C) and washed with an equal volume of sterile 0.1 M phosphate buffer (pH 6.5). The pellet was re-suspended in 10 mL 100 mM NaCl, pre-adjusted to pH 2.0 with 1 M HCl, and stirred for 1 h at 4 °C. Cells were harvested ($3000 \times g$, 30 min, 4 °C) and the cell-free supernatant adjusted to pH 7.0 with sterile 1 M NaOH. BacST8KF activity was tested as described above.

2.8. Effect of medium components of bacST8KF production

Strain ST8KF (100 μL of a 24 h-old culture) was inoculated into 10 mL MRS broth (De Man, Rogosa, & Sharpe, 1960), modified as indicated in Table 3. In the first set of experiments, glucose was replaced with either 20.0 g L^{-1} fructose, 20.0 g L^{-1} lactose, 20.0 g L^{-1} mannose, 20.0 g L^{-1} maltose, or 20.0 g L^{-1} saccharose. In the next set of experiments, the meat extract and yeast extract in MRS broth (De Man et al., 1960) were replaced with either 20.0 g L^{-1} tryptone (Oxoid), 20.0 g L^{-1} meat extract (Biolab), 20.0 g L^{-1} yeast extract (Biolab), 12.5 g L^{-1} tryptone (Oxoid) plus 7.5 g L^{-1} meat extract (Biolab), 12.5 g L^{-1} tryptone (Oxoid) plus 7.5 g L^{-1} yeast extract (Biolab), or 10 g L^{-1} meat extract (Biolab) plus 10 g L^{-1} yeast extract (Biolab). In a third set of experiments, strain ST8KF (100 μL of a 24 h-old culture) was also inoculated into 10 mL MRS broth (De Man et al., 1960), modified by excluding magnesium sulphate, manganese sulphate and tri-ammonium citrate, respectively. In a fourth set of experiments, MRS broth (De Man et al., 1960) was supplemented with the following: glycerol at 1.0, 2.0, 5.0 or 10.0 g L^{-1}, respectively; KH_2PO_4 at 5.0, 10.0 and 20.0 g L^{-1}, respectively; K_2HPO_4 at 5.0, 10.0 and 20.0 g L^{-1}, respectively; cyanocobalamin (vitamin B_{12}) (2.0 mg L^{-1}); thiamine (vitamin B_1) (2.0 mg L^{-1}); L–ascorbic acid (vitamin C) (2.0 mg L^{-1}); DL–6,8–thioctic acid (2.0 mg L^{-1}); and tri-ammonium citrate (5.0 and 10.0 g L^{-1}, respectively). MRS broth (Biolab) was adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.5, respectively; with 1 M NaOH or 1 M HCl. All cultures were incubated at 30 °C for 24 h. BacST8KF activity was tested against *Lb. casei* LHS as before.

3. Results and discussion

3.1. Spectrum of antimicrobial activity

Thirty-five of the 48 isolates from kefir and kefir grains inhibited the growth of *Lb. casei* LHS. From these, the isolate with the strongest antimicrobial activity (isolate ST8KF) was screened against a panel of sensitive strains (Table 1). Cell-free supernatant, adjusted to pH 6.0, inhibited the growth of *Enterococcus mundtii*, *Lb. curvatus*,

Table 1
Spectrum of antimicrobial activity of bacST8KF

Target strain	Growth temperature (in °C)	BacST8KF activity
Enterococcus faecalis 21, BFE 1071, FA2	37	–
Enterococcus faecalis FAIRE 77, FAIRE 88, FAIRE 90, FAIRE 92	37	–
Enterococcus mundtii ST4SA	30	+
Escherichia coli 40, RPEC 1	37	–
Klebsiella pneumoniae 30, 31, 39	37	–
Lactobacillus casei LHS	30	+
Lactobacillus curvatus DF38	30	+
Lactobacillus paracasei subsp. *Paracasei* ST11BR	30	–
Lactobacillus plantarum 423, AMA-K, ST8SH	30	–
Lactobacillus sakei DSM20017[a]	30	–
Lactobacillus salivarius 241	30	+
Lactococcus lactis susbp. *lactis* HV219	30	–
Listeria innocua F, LMG 13568	37	+
Staphylococcus aureus 36, RPSA1	37	–
Streptococcus agalactiae RPSAG 39, RPSAG 48	37	–
Streptococcus sp. TL1	30	–
Streptococcus caprinus ATCC 700065, ATCC 700066	30	–
Streptococcus pneumoniae 29	37	–
Pseudomonas sp. 25	37	–

[a]DSM = Deutsche Sammlung von Cellkulturen und Mikroorganismen, ATCC = American Type Culture Collection. Lactic acid bacteria were cultured in MRS (Biolab) medium and all other bacteria in BHI (Biolab) medium. +, inhibition zone of at least 5 mm in diameter, –, no inhibition zone recorded.

Lb. salivarius and *L. innocua*, but none of the other strains included in the test panel (Table 1). This narrow-spectrum of activity is unique for a bacteriocin produced by *Lb. plantarum*. Most of the bacteriocins described for *Lb. plantarum* are active against a much broader range of genera and species (De Vuyst & Vandamme, 1994).

3.2. Identification of isolate ST8KF

Isolate ST8KF is rod-shaped and based on sugar fermentation reactions (not shown), 99.9% related to *Lb. plantarum*. Amplification of genomic DNA with species-specific primers produced a 350 bp fragment, which corresponded in size to that of *Lb. plantarum* ATCC 14917^T (Fig. 1). The same DNA amplified with primers specific for *Lb. pentosus* and *Lb. paraplantarum*, and DNA from *Lb. paraplantarum* ATCC 700211^T and *Lb. pentosus* ATCC 8041^T amplified with primers specific for *Lb. plantarum* yielded no fragments (Fig. 1). The 16S rDNA amplified from isolate ST8KF revealed 99% homology to the 16S rDNA sequence of *Lb. plantarum* (GenBank accession number AY383631.1). Isolate ST8KF is thus regarded a strain of *Lb. plantarum*.

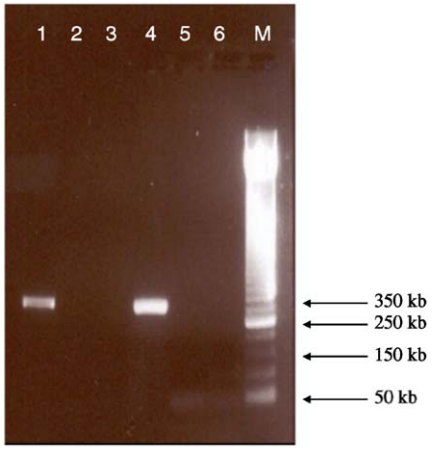

Fig. 1. Agarose gel (1%, w/v) showing DNA fragments obtained after PCR amplification with primers specific for *Lb. plantarum*. (1) Strain ST8KF, (2) *Lb. pentosus* ATCC 8041^T, (3) *Lb. paraplantarum* ATCC 700211^T, (4) *Lb. plantarum* ATCC 14917^T, (5) and (6) no DNA, (M) 50 kb molecular weight marker from Amersham (Bioscience, UK Limited, Buckinghamshire, UK).

3.3. Isolation of bacteriocin ST8KF

According to tricine-SDS–PAGE, bacST8KF is approximately 3.5 kDa in size (Fig. 2). This is within the size range of most bacteriocins reported for the genus *Lactobacillus* (De Vuyst & Vandamme, 1994) and bacteriocins ST26MS and ST28MS described for *Lb. plantarum* (Todorov & Dicks, 2005b). BacST8KF is, however, much smaller than bacteriocin ST13BR (10.0 kDa) and bacteriocin ST194BZ (14.0 kDa) produced by *Lb. plantarum* ST13BR and ST194BZ, respectively (Todorov & Dicks, 2005a; Todorov, Van Reenen, & Dicks, 2004).

3.4. Effect of enzymes, pH, detergents and temperature on bacST8KF

The activity of bacST8KF was destroyed after treatment with Proteinase K and pronase, but not when treated with α-amylase (Table 2). This suggested that the activity of bacST8KF is not dependent on glycosylation. Similar

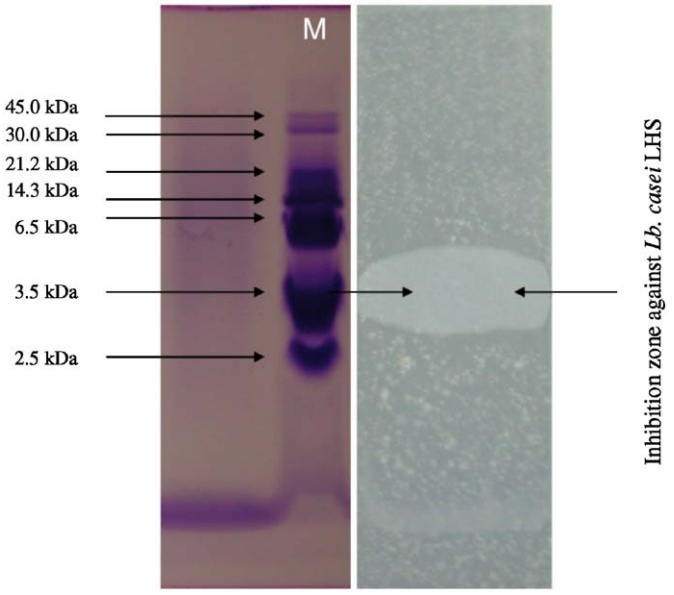

Fig. 2. Separation of bacST8KF by SDS–PAGE. Inhibition of *Lb. casei* LHS is indicated by the arrow. M = low molecular-mass rainbow marker (Amersham Bioscience Europe GmbH).

Table 2
Effect of enzymes, pH, detergents and temperature on bacST8KF

Component		Bacteriocin activity
Enzyme	α-amylase	+[a]
	Proteinase K	−
	Pronase	−
PH	2.0–10.0	+
Temperature	30 °C	+
	45 °C	+
	60 °C	+
	100 °C	+
	121 °C	+
Detergents	Triton X100, Triton X114	−
	Tween 20, Tween 80	+
	SDS, Urea	+
Protease inhibitor	EDTA	+

[a] +, inhibition zone of at least 5 mm in diameter; −, no inhibition zone recorded.

results have been reported for other bacteriocins of *Lb. plantarum* (DeVuyst & Vandamme, 1994; Kelly, Asmundson, & Huang, 1996; Todorov & Dicks 2004b). Leuconocin S, produced by *Leuconostoc paramesenteroides* (Lewus, Sun, & Montville, 1992) and carnocin 54, produced by *Leuc. carnosum* (Keppler, Geisen, & Holzapfel, 1994) are examples of amylase-sensitive bacteriocins.

BacST8KF remained stable after incubation (30 °C) at pH 2.0, 4.0, 6.0, 8.0 and 10.0 (Table 2). No bacST8KF activity was recorded after treatment with Triton X100 and Triton X114, respectively (Table 2). However, treatment with Tween 20, Tween 80, SDS, urea and EDTA had no effect on the activity of bacST8KF (Table 2). Similar results were reported for bacteriocin J46 produced by *Lc. lactis* subsp. *cremoris* (Hout, Maghrous, & Barena-Gonzalez, 1996). However, plantaricin C19 produced by *Lb. plantarum* C19 lost its activity after treatment with SDS or Triton X-100 (Atrih, Rekhif, Moir, Lebrihi, & Lefebvre, 2001). Treatment of enterocin EJ97 produced by *Enterococcus faecalis* EJ97 (Gálvez, Valdivia, & Abriouel, 1998), bozacin B14 produced by *Lc. lactis* subsp. *lactis* B14 (Ivanova, Kabadjova, Pantev, Danova, & Dousset, 2000) and pediocin ST18 produced by *Pediococcus pentosaceus* ST18 (Todorov & Dicks, 2005c) with SDS did not result in any activity loss.

BacST8KF (pH 5.5) remained active after 20 min at 121 °C. Similar results were recorded for a number of bacteriocins produced by *Lactobacillus* and *Lactococcus* spp. (Klaenhammer, 1988; Ko & Ahn, 2000; Todorov & Dicks, 2004b; Todorov & Dicks, 2005a–c; Van Reenen et al., 1998). Moreover, lactocin NK24, produced by *Lc. lactis* NK24, lost 87.5% of its activity after 30 min at 100 °C and was completely inactivated after 15 min at 121 °C (Lee and Paik, 2001). In the case of lactocin MMFII produced by *Lc. lactis* MMFII, only 8.3% activity was recorded after 30 min at 110 °C and 25% after 30 min at 80 and 90 °C (Ferchichi, Frere, Mabrouk, & Manai, 2001). Nisin, produced by *Lc. lactis* subsp. *lactis* WNC20, was inactivated after 15 min at 121 °C when incubated at pH 7.0, but not when incubated at pH 3.0 (Noonpakdee, Santivarangkna, Jumriangrit, Sonomoto, & Panyim, 2003). Bozacin B14, produced by *Lc. lactis* subsp. *lactis* B14, was inactivated after 10 min at 90 °C (Ivanova et al., 2000).

3.5. Production of bacST8KF

The cell density of *Lb. plantarum* ST8KF increased from 0.3 to 10.0 (OD_{600}) during 30 h of growth at 30 °C (Fig. 3). The pH decreased from 6.10 to 3.60 over the same period (Fig. 3). Production of bacST8KF increased from 800 AU mL^{-1} after 3 h of growth to 12,800 AU mL^{-1} during the following 6 h (Fig. 3). Production remained at 12,800 AU mL^{-1} for at least 12 h and increased to 51,200 AU mL^{-1} in the next 6 h (Fig. 3). Production was still at 51,200 AU mL^{-1} after 27 h. Optimal production of bacST8KF was recorded during stationary growth, which may suggest that the peptide is a secondary metabolite. Similar results were reported for plantaricin ST31 (Todorov et al., 1999), bacteriocin ST26MS and bacteriocin ST28MS (Todorov & Dicks, 2005b). This is contrary to other bacteriocins thus far described for *Lb. plantarum* (Todorov & Dicks, 2004b; Todorov et al., 2004; Van Reenen et al., 1998). The pH of the culture decreased from 6.2 to approximately 4.1 during the first 9 h of fermentation (Fig. 3). The pH decreased to approximately 3.7 during the following 12 h and to 3.60 during the period of maximum production of bacST8KF (Fig. 3). These results show that bacST8KF is stable at pH 3.6, as determined with pH stability tests (Table 2). The sudden increase in activity from 12,800 to 51,200 AU mL^{-1} occurred at pH 3.7 and 3.6 (Fig. 3) and cannot be ascribed to a change in culture pH. It is unlikely that such a small

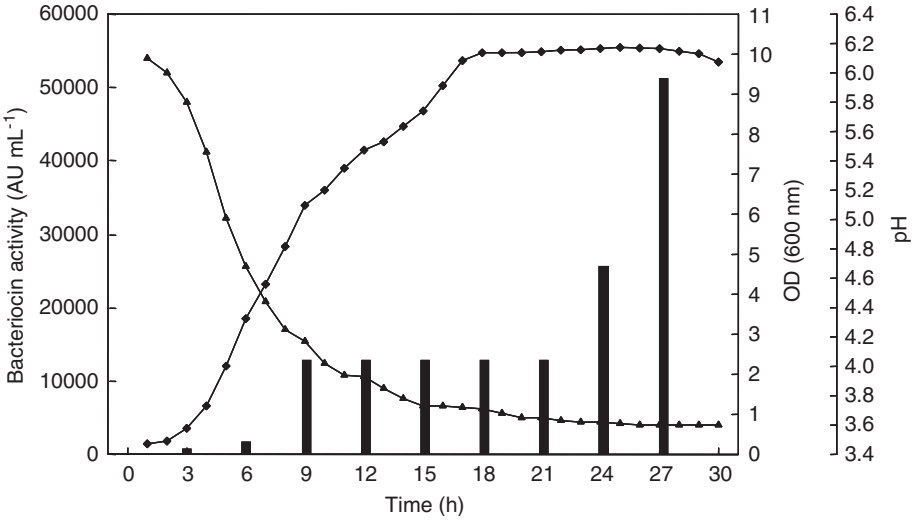

Fig. 3. Growth of *Lb. plantarum* ST8KF and bacST8KF production in MRS broth (Biolab). Symbols: (♦) = growth, (▲) = change in pH, (■) = bacST8KF production. Incubation was at 30 °C.

change in pH could trigger a sudden release of bacST8KF from the surface of the producer cell, as reported by Yang, Johnson, and Ray (1992) and Van Reenen et al. (1998). The increase in activity could be due to the metabolism of remaining nutrients or medium component(s) not required for cell growth.

3.6. Effect of medium components on bacST8KF production

BacST8KF was produced at 25600 AU mL^{-1} when strain ST8KF was grown in MRS broth supplemented with 20 g L^{-1} glucose (Fig. 3, Table 3). The same level of activity was recorded when glucose was replaced by 20 g L^{-1} saccharose (Table 3). Fructose as sole carbon source, on the other hand, yielded only 200 AU mL^{-1}, suggesting that the glucose moiety of sucrose is favored for production (Table 3). Low levels of activity were recorded when the cells were grown in the presence of the same concentration of mannose (200 AU mL^{-1}) and lactose (6400 AU mL^{-1}). No activity was recorded when the cells were grown in the presence of 20 g L^{-1} maltose, suggesting that it may have a regulatory function.

Our results show that bacST8KF production is stimulated by glucose and sucrose. Similar results have been reported for plantaricin UG1 (Enan, Essawy, Uyttendaele, & Debevere, 1996), plantaricin KW30 (Kelly et al., 1996) and plantaricin ST31 (Todorov, Gotcheva, Dousset, Onno, & Ivanova, 2000).

Of all nitrogen sources tested (Table 3), meat extract yielded the highest activity (12800 AU mL^{-1}). A combination of meat extract and tryptone (7.5:12.5 g L^{-1}), or meat extract and yeast extract (10.0:10.0 g L^{-1}) yielded 6400 AU mL^{-1} (Table 3), suggesting that meat extract in

Table 3
Effect of carbohydrates, nitrogen, potassium, glycerol, vitamins and tri-ammonium sulphate on bacST8KF production

Component	Concentration (g L^{-1})	BacST8KF activity (AU mL^{-1})
Glucose	20.0	25600
Fructose	20.0	200
Lactose	20.0	6400
Mannose	20.0	200
Maltose	20.0	0
Saccharose	20.0	25600
Tryptone	20.0	3200
Meat Extract	20.0	12800
Yeast Extract	20.0	1600
Tryptone and Meat Extract	12.5 and 7.5	6400
Tryptone and Yeast Extract	12.5 and 7.5	1600
Meat Extract and Yeast Extract	10 and 10	6400
Glycerol	1.0	12800
	2.0	12800
	5.0	6400
	10.0	3200
	20.0	0
KH_2PO_4	5.0	6400
	10.0	25600
	20.0	1600
K_2HPO_4	5.0	3200
	10.0	800
	20.0	3200
Cyanocobalamin (B12)	0.002	12800
Thiamine (B1)	0.002	12800
L-ascorbic acid (C)	0.002	3200
DL-6,8thioctic acid	0.002	6400
Tri-ammonium citrate	0	25600
	5.0	12800
	10.0	6400

excess of $10\,g\,L^{-1}$ is required for optimal bacteriocin production. BacST8KF production was not stimulated by yeast extract (Table 3). These results are contradictory to those reported for plantaricin 423 produced by *Lb. plantarum* 423. Highest production of plantaricin 423 was obtained in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract (Verellen, Bruggeman, Van Reenen, Dicks, & Vandamme, 1998). Stimulation of bacteriocin production by meat extract has been reported for pediocin AcH (Bhunia, Kim, Johnson, & Ray, 1998) and helveticin J (Joerger and Klaenhammer, 1986).

MRS medium, supplemented with $1.0\,g\,L^{-1}$ and $2.0\,g\,L^{-1}$ glycerol yielded higher levels of bacST8KF ($12{,}800\,AU\,mL^{-1}$) compared with 5.0 and $10.0\,g\,L^{-1}$ glycerol (Table 3). No bacST8KF activity was recorded in the presence of $20.0\,g\,L^{-1}$ glycerol (Table 3). Similar results have been reported for plantaricin ST31, in which case glycerol at $2.0\,g\,L^{-1}$ and higher resulted in lower activity (Todorov et al., 2000). Glycerol is not used as a carbon source and the decrease in bacteriocin production may be due to changes in osmotic stress (Todorov & Dicks, 2005a). This merits further research.

Little is known about the influence of potassium ions on the production of bacteriocins (Todorov & Dicks, 2004a). Levels of $10.0\,g\,L^{-1}$ KH_2PO_4 yielded optimal levels of bacST8KF ($25{,}600\,AU\,mL^{-1}$), while the same level of K_2HPO_4 yielded only $800\,AU\,mL^{-1}$ (Table 3). The increase in activity cannot be due to pH changes caused by higher potassium levels, since all media were adjusted to pH 6.5 before inoculation. In the case of plantaricin UG1, $7.0\,g\,L^{-1}$ K_2HPO_4 resulted in increased activity (Enan et al., 1996). The optimal level of K_2HPO_4 recorded for plantaricin ST31 was between 2.0 and $5\,g\,L^{-1}$ (Todorov et al., 2000). On the other hand, no difference in antibacterial activity was recorded when *Lb. plantarum* ST194BZ was grown in the presence of $2.0\,g\,L^{-1}$ KH_2PO_4 and $2.0\,g\,L^{-1}$ K_2HPO_4.

BacST8KF production was stimulated by cyanobalamin and thiamine ($12{,}800\,AU\,mL^{-1}$), but not by L–ascorbic acid ($3200\,AU\,mL^{-1}$) and DL–6,8–thioctic acid ($6400\,AU\,mL^{-1}$) (Table 3). Similar results have been reported for bacteriocin ST13BR (Todorov et al., 2004) and bacteriocin ST194BZ (Todorov & Dicks, 2005a).

BacST8KF production was lower in the presence of 5.0 and $10.0\,g\,L^{-1}$ tri-ammonium citrate (12,800 and $6{,}400\,AU\,mL^{-1}$, repectively), compared with growth in its absence ($25{,}600\,AU\,mL^{-1}$) (Table 3).

Bacteriocin production of $6{,}400\,AU\,mL^{-1}$ was observed in the absence of magnesium sulphate, while no bacteriocin production was observed in the absence of manganese sulphate (data not shown). Therefore, manganese sulphate is required for bacteriocin production, and magnesium sulphate has a stimulatory effect on bacST8KF.

BacST8KF was not produced in MRS broth with an initial pH of 4.5 while $3200\,AU\,mL^{-1}$ were produced at a media pH of 5.0. Bacteriocin production of $25{,}600\,AU\,mL^{-1}$ was observed for all other pH media tested (pH 5.5, 6.0, 6.5). Similar results were reported for other bacteriocins produced by *Lb. plantarum* (Daeschel, McKeney, & Mac Donald, 1990; Kelly et al., 1996; Todorov & Dicks, 2005b; Todorov et al., 2000). From these results and literature data (Daeschel et al., 1990; Kelly et al., 1996; Todorov & Dicks, 2005b; Todorov et al., 2000) it can be concluded that an initial pH of 5.5 or greater is required for optimal growth of *Lb. plantarum* ST8KF and bacteriocin production.

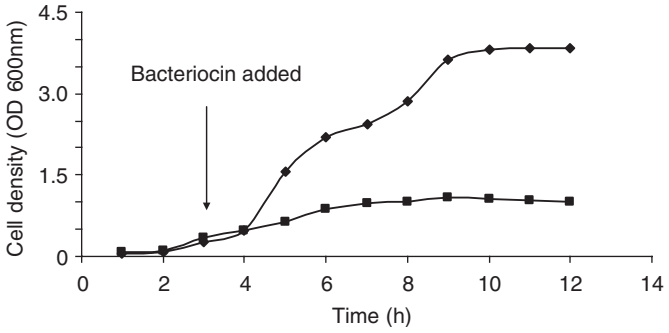

Fig. 4. Effect of bacST8KF on the growth of *Lb. casei* LHS over a period of 12 h (■). The control (◆) received no bacST8KF.

3.7. Mode of activity

Lb. casei LHS treated with bacST8KF ($12{,}800\,AU\,mL^{-1}$) increased from OD_{600} 0.3 to 1.0 over 9 h (Fig. 4). The control (not treated with bacST8KF) increased from OD_{600} 0.3 to 4.0 over the same period (Fig. 4). The slight increase in optical density of cells treated with bacST8KF suggests that the mode of activity is bacteriostatic. The cell density of treated cells remained more-or-less the same ($OD\ 600_{\ nm} = 1.2$) for the last 3 h of growth, which suggests that the cells do not recover from the treatment. Similar results were reported for bacteriocins ST11BR, ST151BR and ST34BR (Todorov & Dicks, 2004b). Bacteriocins ST28MS and ST26MS, on the other hand, repressed the growth of strain LHS for only 2 h (Todorov & Dicks, 2005b).

3.8. Adsorption of bacST8KF to producer cells

Low bacST8KF activity was detected after treatment of 18 h-old cells of strain ST8KF with 100 mM NaCl. The activity was, however lower than the activity recorded in the cell-free supernatant, suggesting that bacST8KF adsorbs to the surface of the producer cells in low concentrations. Similar results were reported for plantaricin C19, maximal adsorption to the producer cells was recorded between pH 5 and 7, with complete loss of adsorption at pH 1.5 and 2.0 (Atrih et al., 2001). In the case of plantaricin ST31 (Todorov et al., 1999), pediocin ST18 (Todorov & Dicks, 2005c) and bozacin B14 (Ivanova et al.,

2000) no bacteriocin activity was recorded on the cell surface of the producer strains.

4. Conclusions

BacST8KF (3.5 kDa) has a narrow spectrum of activity, is heat resistant and stable between pH 2.0 and 10.0, adsorbs to the surface of the producer cell in low concentrations and is produced at 51,200 AU mL^{-1} after 27 h of fermentation in the presence of 2% (w/v) D-glucose. Low activity levels (6,400 AU mL^{-1}) have been recorded in the presence of lactose, and in the presence of citrate (tri-ammonium citrate), suggesting that the bacteriocin may not be produced at high levels during the initial phases of kefir production. However, bacST8KF production may increase towards the end of the production process when the citrate has been consumed by the rest of the microflora. Strain ST8KF may be used in a mixed starter culture. Further research on the production of specific flavor compounds is in progress.

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