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Optimization of bacteriocin production by *Lactobacillus plantarum* ST13BR, a strain isolated from barley beer

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The cell-free supernatant containing bacteriocin ST13BR, produced by *Lactobacillus plantarum* ST13BR, inhibits the growth of *L. casei*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Escherichia coli*. Based on tricine-SDS-PAGE, bacteriocin ST13BR is 10 kDa in size. Complete inactivation or significant reduction in bacteriocin activity was observed after treatment with Proteinase K, trypsin and pronase, but not with catalase or α -amylase. Low bacteriocin activity (200 AU/ml) was recorded in BHI medium, M17 broth, 10% (w/v) soy milk, and 2% and 10% (w/v) molasses, despite good growth. Maximal bacteriocin activity (6,400 AU/ml) was recorded after 23 h in MRS broth, but only at 30°C. Tween 80 in MRS broth increased bacteriocin production by more than 50%. Meat extract or yeast extract as sole nitrogen source, or a combination of the two (1:1) in MRS broth, stimulated bacteriocin production (6,400 AU/ml). Only 50% activity (3,200 AU/ml) was recorded with tryptone as sole nitrogen source, whereas a combination of tryptone, meat extract and yeast extract yielded 6,400 AU/ml. Bacteriocin production was not stimulated by the addition of glucose at 2.0% w/v (3,200 AU/ml), nor 2% (w/v) fructose, sucrose, lactose or mannose, respectively (800 AU/ml). Activity levels less than 200 AU/ml were recorded in the presence of 0.05% to 0.5% (w/v) maltose. Maximal bacteriocin production (6,400 AU/ml) was recorded in the presence of 2% (w/v) maltose. Maltose at 4.0% (w/v) led to a 50% reduction of bacteriocin activity. The presence of 1.0% (w/v) and higher KH_2PO_4 , or glycerol at 0.2% (w/v) suppressed bacteriocin production.

Key Words—bacteriocin ST13BR; *Lactobacillus plantarum*; traditional barley beer

Introduction

Lactobacillus plantarum has been isolated from various habitats and bacteriocins (antimicrobial peptides) have been described for strains from fermented meat products (Garriga et al., 1993; Schillinger and Lücke, 1989), milk (Rekhif et al., 1995), cheese (Gonzales et al., 1994), fermented cucumber (Atrih et al., 1993; Daeschel et al., 1990), olives (Jimenez-Diaz et al., 1993), dough (Gobetti et al., 1994; Infantes and

Tourneur, 1991), silage (Beck, 1978), pineapple (Kato et al., 1994), grapefruit juice (Kelly et al., 1996) and sorghum beer (Van Reenen et al., 1998).

Apart from studies conducted on the effect of nitrogen and carbon sources on the production of plantaricin ST31 (Todorov et al., 2000), plantaricin 423 (Verellen et al., 1998), plantaricin UG1 (Enan et al., 1996), plantaricin KW30 (Kelly et al., 1996), plantaricin-149 (Kato et al., 1994), plantaricin S (Jimenez-Diaz et al., 1990) and plantaricin A (Daeschel et al., 1990), little is known about the growth conditions required for optimal production of bacteriocins by *L. plantarum*. Studies conducted on bacteriocins from other lactic acid bacteria, e.g. pediocin AcH (Biswas et al., 1991), pediocin PD-1 (Nel et al., 2001), enterocin

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1146 (Parente and Ricciardi, 1994), enterocin AS-48 (Abriouel et al., 2001), enterocin P (Herranz et al., 2001), sakP (Aasen et al., 2000), and bacteriocins produced by *Leuconostoc mesenteroides* L124 (Matargas et al., 2003) have shown that production is often regulated by growth pH and temperature. In some cases, higher bacteriocin activity has been recorded at sub-optimal growth conditions (Aasen et al., 2000; Audisto et al., 2001; Bogovic-Matijasic and Rogelj, 1998; De Vuyst et al., 1996; Kim et al., 1997; Krier et al., 1998; Matsusaki et al., 1996; Mortvedt-Abildgaard et al., 1995; Parente and Ricciardi, 1994; Parente et al., 1994; Todorov et al., 2000).

In this paper we report on bacteriocin ST13BR from *L. plantarum* ST13BR, a strain isolated from traditionally fermented barley beer produced in South Africa. The effects of nutrients and medium pH on the activity levels of bacteriocin ST13BR are described.

Materials and Methods

Bacterial strains and growth media. Strain

ST13BR was isolated from barley beer produced by the fermentation of maize, barley, soy flour and sugar and was classified as *L. plantarum* based on physiological and biochemical characteristics described by Kandler and Weiss (1986), Schillinger and Lücke (1987), Cogan et al. (1997) and Stiles and Holzapfel (1997). Sugar fermentation reactions were confirmed by the API 50 CHL system (Biomérieux, Marcy-l'Etoile, France).

Strain ST13BR was grown in MRS medium (Biolab, Biolab Diagnostics, Midrand, South Africa) at 30°C. The growth media, incubation temperature and origin of the strains included in this study are listed in Table 1. Strains were stored at -80°C in spent MRS broth in the presence of 15% (v/v) glycerol. MRS medium (Biolab) was used for all experiments, except for carbon and nitrogen optimization, in which case MRS (De Man et al., 1960) was used.

Bacteriocin bioassay. Bacteriocin screening was performed by using the agar-spot test and the well diffusion method, as described by Schillinger and Lücke (1989) and Tagg and McGiven (1971), respectively.

Table 1. Growth medium and incubation temperature of indicator strains, and inhibitory spectrum of the cell-free supernatant of strain ST13BR.

Indicator strain	Medium and temperature (°C)	Origin	Bacteriocin activity
<i>Escherichia coli</i> 40	BHI, 37	Human middle ear	+
<i>Escherichia coli</i> 8	BHI, 37	Mastitic milk	+
<i>Enterobacter cloacae</i> 24	BHI, 37	Human middle ear	-
<i>Enterococcus faecalis</i> 20; 21	MRS, 37	Human middle ear	+
<i>Enterococcus faecalis</i> E77; E80; E90; E92; FA2	MRS, 37	Pig feces	-
<i>Klebsiella pneumoniae</i> 31	BHI, 37	Human middle ear	+
<i>Lactobacillus casei</i> LHS	BHI, 37	Wine	++
<i>Lactobacillus curvatus</i> DF38	MRS, 30	Salami	-
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	MRS, 30	Yoghurt	-
<i>Lactobacillus plantarum</i> 423	MRS, 30	Sorghum beer	-
<i>Lactobacillus plantarum</i> LMG 13556	MRS, 37	Silage	-
<i>Lactobacillus sakei</i> DSM 20017	MRS, 30	Sake	-
<i>Lactobacillus salivarius</i> 241	MRS, 30	Pig intestine	-
<i>Listeria innocua</i> F LMG 13568	BHI, 37	Meat	-
<i>Pseudomonas aeruginosa</i> 7	BHI, 37	Human middle ear	+
<i>Staphylococcus aureus</i> 2	BHI, 37	Human middle ear	-
<i>Staphylococcus uberis</i> 12	BHI, 37	Mastitic milk	-
<i>Streptococcus agalactiae</i> 9	BHI, 37	Mastitic milk	-
<i>Streptococcus caprinus</i> ATCC 700065; ATCC 700066	BHI, 37	Goat feces	-
<i>Streptococcus pneumoniae</i> 4	MRS, 37	Human middle ear	-
<i>Streptococcus</i> sp. TL1; TL2R; TL2W	MRS, 37	Goat intestine	-

+, inhibition zone less than 10 mm in diameter; ++, inhibition zone larger than 10 mm in diameter; -, no inhibition zone recorded.

Adjustment of the cell-free supernatant to pH 6.0 with 1 N NaOH prevented the inhibitory effect of lactic acid. Antimicrobial activity was expressed as arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (Van Reenen et al., 1998). The indicator strains are listed in Table 1.

Molecular size of bacteriocin ST13BR. Strain ST13BR was grown in MRS broth for 20 h at 30°C. The cells were harvested by centrifugation (8,000×g, 10 min, 4°C) and the bacteriocin precipitated from the cell-free supernatant with 40% ammonium sulphate. The precipitate was resuspended in one tenth volume of 25 mM ammonium acetate (pH 6.5) and then desalted by using a 1,000 Da cut-off dialysis membrane (Spectrum, Inc., CA, USA). Peptides were separated by tricine-SDS-PAGE, as described by Schagger and Von Jagow (1987). A low molecular weight marker with sizes ranging from 2.35 to 46 kDa (Amersham International, UK) was used. The gels were fixed and one half stained with Coomassie Blue R250 (Saarchem, Krugersdorp, South Africa) and the position of the active bacteriocin determined on an unstained gel, as described by Van Reenen et al. (1998). *Lactobacillus casei* LHS (10⁶ cfu/ml), suspended in Brain Heart Infusion broth (BHI, Biolab), supplemented with 1.0% agar (w/v), was used as a sensitive strain.

Effect of enzymes on bacteriocin ST13BR. Strain ST13BR was grown in MRS broth at 30°C for 24 h, the cells harvested by centrifugation (8,000×g, 10 min, 4°C), and the cell-free supernatant adjusted to pH 6.0 with 6 N NaOH. One milliliter cell-free supernatant was incubated for 2 h in the presence of 1 mg/ml and 0.1 mg/ml Proteinase K (Roche, Indianapolis, IN, USA), pronase and trypsin (Boehringer Mannheim GmbH, Germany), α-amylase (Sigma Diagnostics, St. Louis, MO, USA) and catalase (Boehringer Mannheim), respectively, according to Ivanova et al. (1998). Antimicrobial activity was recorded by using the agar-spot test method as described before.

Production of bacteriocin ST13BR in different growth media and at different initial growth pH. An 18-h-old culture of strain ST13BR was inoculated (2%, v/v) into MRS broth, BHI broth, M17 broth (Merck, Darmstadt, Germany), soy milk (10%, w/v, soy flour) and molasses (2 to 10%, w/v, with 2% intervals), respectively. Incubation was at 30°C and 37°C, respectively, without agitation, for 28 h. Samples were taken every hour and examined for bacterial growth (OD at 600 nm),

changes in culture pH, and antimicrobial activity (AU/ml) against *L. casei* LHS. The agar-spot test method was used as described before.

In a separate experiment, the effect of initial medium pH on bacteriocin ST13BR production was tested. Volumes of 300 ml MRS broth were adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.5, respectively, with 6 N HCl or 6 N NaOH and then autoclaved. Each flask was inoculated with 2% (v/v) of an 18-h-old culture of strain ST13BR and incubated at 30°C for 20 h, without agitation. Changes in culture pH and production of bacteriocin ST13BR (AU/ml) were determined every hour, as described before.

Effect of medium composition on bacteriocin ST13BR production. Strain ST13BR was grown in 10 ml MRS broth for 18 h at 30°C, the cells harvested by centrifugation (8,000×g, 10 min, 4°C), and the pellet resuspended in 10 ml sterile peptone water. Four milliliters of this cell suspension was used to inoculate 200 ml of the following media: (a) MRS broth (De Man et al., 1960), without organic nutrients, supplemented with tryptone (20 g/L), meat extract (20 g/L), yeast extract (20 g/L), tryptone (12.5 g/L) plus meat extract (7.5 g/L), tryptone (12.5 g/L) plus yeast extract (7.5 g/L), meat extract (7.5 g/L) plus yeast extract (7.5 g/L), or a combination of tryptone (10.0 g/L), meat extract (5.0 g/L) and yeast extract (5.0 g/L), respectively; (b) MRS broth, i.e. with 2% (v/v) glucose; (c) MRS broth without glucose, supplemented with 2% (v/v) fructose, sucrose, lactose, mannose, and maltose, respectively; (d) MRS broth with 0.05–4.0% (w/v) maltose as sole carbon source; (e) MRS broth with 2 g/L K₂HPO₄ or 2–50 g/L KH₂PO₄; and (f) MRS broth supplemented with 0–50 g/L glycerol. In a separate experiment, the vitamins cyanocobalamin (Sigma, St. Louis, MO, USA), L-ascorbic acid (BDH Chemicals Ltd., Poole, UK), thiamine (Sigma), DL-6,8-thioctic acid (Sigma) and Vitamin K₁ (Fluka Chemie AG, CH-9471 Buchs, Switzerland) were filter-sterilized and added to MRS broth at 1 mg/ml (final concentration). Incubation for all tests was at 30°C for 20 h. Activity levels of bacteriocin ST13BR were determined as described before.

Results and Discussion

The cell-free supernatant of strain ST13BR inhibited the growth of *L. casei*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Esch-*

erichia coli, but not that of the other species included in the study (Table 1). Activity against Gram-negative bacteria is an unusual phenomenon and has thus far only been reported for thermophilin 81, produced by *Streptococcus thermophilus*, bacteriocins produced by *L. paracasei* subsp. *paracasei* L126 and L134, a bacteriocin produced by *L. lactis* KCA2386, and plantaricin 35 d, produced by *L. plantarum* (Caridi, 2002; Ivanova et al., 1998; Ko and Ahn, 2000; Messi et al., 2001).

According to tricine-SDS-PAGE, bacteriocin ST13BR has a molecular mass of approximately 10 kDa (Fig. 1), which is larger than most bacteriocins thus far described for *L. plantarum* (De Vuyst and Vandamme, 1994). Complete inactivation or significant reduction in antimicrobial activity was observed after treatment of the cell-free supernatant with Proteinase K, pronase (Fig. 2) and trypsin. Treatment with catalase resulted in no activity change (Fig. 2), indicating that H_2O_2 was not responsible for inhibition. Treatment with α -amylase did not alter the antimicrobial activity (Fig. 2), suggesting that bacteriocin ST13BR is not glycosylated and thus similar to most other bacteriocins (De Vuyst and Vandamme, 1994). Leuconocin S, produced by *Leuconostoc paramesenteroides* (Lewus et al., 1992) and carnocin 54, produced by *Leuconostoc carnosum* (Keppler et al., 1994) are typical examples of amylase-sensitive bacteriocins.

Low bacteriocin activity (200 AU/ml) was recorded when strain ST13BR was grown in BHI and M17 broth, despite good growth. Similar results were recorded in the presence of 10% (w/v) soy milk (results not shown). Good growth was also recorded in 2% to 10% (w/v) molasses, but low levels of bacteriocin were produced (200 AU/ml). The low activity levels recorded in M17 broth, BHI broth, soy milk and molasses, despite relatively good growth, suggests that specific nutrients are required for bacteriocin production. Furthermore, with bacteriocin production recorded at 30°C and not at 37°C, growth temperature seems to play an important role. Growth temperature and bacteriocin production are often correlated, as observed for lactocin A (Parente et al., 1994), enterocin 1146 (Parente and Ricciardi, 1994), lactocin S (Mortvedt-Abildgaard et al., 1995), amylovorin 1471 (De Vuyst et al., 1996) and nisin Z (Matsusaki et al., 1996).

The highest yield of bacteriocin ST13BR activity (6,400 AU/ml) was recorded after 23 h in MRS broth (Biolab), and only when incubated at 30°C. During 28 h of growth, the pH decreased from 6.0 to 3.6 (Fig.

Fig. 1. Tricine-SDS-PAGE of bacteriocin ST13BR.

Lane a: molecular weight marker. Lane b: peptide band stained with Coomassie Blue R250. Lane c: zone of growth inhibition, corresponding to the position of the peptide band in lane b. The gel was covered with viable cells of *L. casei* LHS (approx. 10^6 cfu/ml), imbedded in MRS agar. Incubation was at 30°C for 24 h.

Fig. 2. Antimicrobial activity of bacteriocin ST13BR.

Positions 1 and 2: cell-free supernatant; 3 and 4: cell-free supernatant treated with α -amylase (0.1 mg/ml); 5: cell-free supernatant treated with catalase (0.1 mg/ml); 6: cell-free supernatant treated with pronase (0.1 mg/ml); and 7: cell-free supernatant treated with Proteinase K (0.1 mg/ml). *L. casei* LHS (approx. 10^6 cfu/ml) was used as sensitive strain.

3) and the cell density increased from 0.2 to 10.0 (Fig. 3). Low levels of bacteriocin activity (approximately 200 AU/ml) were recorded after 5 h of growth in MRS broth, suggesting that the peptide is a primary metabolite. Similar results were reported for plantaricin Y (Chin et al., 2001) and bacteriocins produced by *P. acidilactici* (Nieto-Lozano et al., 2002), and *L. paracasei* subsp. *paracasei* M3 (Atanassova et al., 2003). Production of bacteriocin ST13BR decreased to 1,600 AU/ml after 23 h of growth (Fig. 3). Similar decreases in activity have been recorded for lactacin B

Fig. 3. Growth of strain ST13BR in MRS broth under nonregulated pH (●), production of bacteriocin ST13BR (■) and changes in culture pH (▲).

Table 2. Influence of initial growth pH on the antimicrobial activity of bacteriocin ST13BR.

Initial pH	4.5	5.0	5.5	6.0	6.5
Final pH	3.4	3.6	3.6	3.7	3.7
Bacteriocin activity (AU/ml)	<200	800	800	3,200	6,400
Reduction of bacteriocin activity (%) ^a	100	88.5	88.5	50	0

^a Based on the highest activity, recorded as 6,400 AU/ml.

(Barefoot and Klaenhammer, 1984), mesenterocin 5 (Daba et al., 1991), helveticin J (Joerger and Klaenhammer, 1986) and enterocin 1146 (Parente et al., 1997). In the latter studies loss of activity has been ascribed to proteolytic degradation, protein aggregation, adsorption to cell surfaces or feedback regulation (Aasen et al., 2000; De Vuyst et al., 1996; Leyeune et al., 1998; Parente and Ricciardi, 1994; Parente et al., 1994). Strain ST13BR does not hydrolyze casein on agar plates, suggesting that it does not produce extracellular proteases. The activity of bacteriocin ST13BR did not decrease further during 48 h of incubation at room temperature (data not shown), suggesting that specific peptidases active against the bacteriocin were not produced. Furthermore, since no cell lysis occurred after 23 h of growth, based on OD readings (Fig. 3), it is safe to assume that loss of activity was not due to degradation by intracellular proteases.

Low levels of bacteriocin ST13BR activity (<200 AU/ml) were recorded in MRS broth adjusted to pH 4.5 (Table 2). In the same medium adjusted to pH 5.0, 5.5, 6.0 or 6.5, higher levels of bacteriocin activity were recorded (800–6,400 AU/ml; Table 2). Similar results were reported for other bacteriocins produced by

L. plantarum (Daeschel et al., 1990; Jimenez-Diaz et al., 1990; Kato et al., 1994; Kelly et al., 1996; Todorov et al., 2000). The final pH of the cultures was similar (3.4 to 3.7; Table 2), irrespective of the initial growth pH. Based on these results and the low activity levels recorded towards the end of fermentation (200 AU/ml; Fig. 3), loss of bacteriocin activity may be ascribed to acid sensitivity. Further research is needed to determine if bacteriocin ST13BR aggregates at low pH (i.e. at pH 3.6).

Inclusion of Tween 80 in the growth medium increased bacteriocin ST13BR production by more than 50% (results not shown). This corresponds to results obtained for plantaricin 423 (Verellen et al., 1998), pediocin ACh (Biswas et al., 1991) and lactocin 705 (Vignolo et al., 1995).

Growth of strain ST13BR in the presence of meat extract or yeast extract as the only nitrogen source or a combination of the two in ratio 1 : 1 resulted in maximal activity (6,400 AU/ml; Fig. 4a). In the presence of tryptone as the only nitrogen source, 3,200 AU/ml bacteriocin was produced. However, when meat extract or yeast extract, or both, were added to tryptone, 6,400 AU/ml bacteriocin was produced. In the case of

Fig. 4. Bacteriocin ST13BR production (AU/ml) in the presence of (a) tryptone (T), meat extract (M), yeast extract (Y), or a combination thereof, glucose (Glu), fructose (Fru), sucrose (Suc), lactose (Lac), mannose (Man) and maltose (Mal) at concentrations listed in the text; (b) different concentrations of maltose, (c) different concentrations of potassium and (d) different levels of glycerol and the vitamins: thiamine (B₁), L-ascorbic acid (C), cyanocobalamin (B₁₂), Vitamin K₁ (K₁) and DL-6,8-thioctic acid (DL).

plantaricin 423, highest production was obtained in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract (Verellen et al., 1998). Stimulation of bacteriocin production by yeast extract and meat extract has also been reported for pediocin AcH (Bhunia et al., 1988) and helveticin J (Joerger and Klaenhammer, 1986).

Growth of strain ST13BR in the presence of 2.0% (w/v) glucose resulted in 3,200 AU/ml bacteriocin (Fig. 4a). Similar results were recorded for sakacin P (Aasen et al., 2000) and enterocin 1146 (Parente et al., 1997), for which glucose levels of 2.0% (w/v) and above reduced bacteriocin production. However, glucose stimulated the production of plantaricin UG1 (Enan et al., 1996), plantaricin 149 (Kato et al., 1994), plantaricin KW30 (Kelly et al., 1996) and plantaricin ST 31 (Todorov et al., 2000).

Growth of strain ST13BR in the presence of 2.0% (w/v) fructose, sucrose, lactose or mannose resulted in low levels of bacteriocin ST13BR activity (800 AU/ml; Fig. 4a). Activity levels of less than 200 AU/ml were

recorded in the presence of 0.05% to 0.5% (w/v) maltose (Fig. 4b). Maltose at 1.0% (w/v) yielded 800 AU/ml, whereas 1.5% (w/v) resulted in bacteriocin production of 1,600 AU/ml, and 2.0% and 3.0% (w/v) maltose in 6,400 AU/ml (Fig. 4b). Maltose at 4.0% (w/v) resulted in a 50% reduction of bacteriocin activity (Fig. 4b).

Little is known about the influence of potassium ions on the production of bacteriocins. A difference in bacteriocin activity was recorded when strain ST13BR was grown in the presence of 0.2% (w/v) KH₂PO₄ and 0.2% (w/v) K₂HPO₄ (Fig. 4c). Concentrations of 0.5% (w/v) and higher KH₂PO₄ repressed bacteriocin activity. In the case of plantaricin UG1, 0.7% (w/v) K₂HPO₄ resulted in increased activity (Enan et al., 1996). The optimal level of K₂HPO₄ recorded for plantaricin ST31 was between 0.2% and 0.5%, w/v (Todorov et al., 2000).

Bacteriocin ST13BR production was the highest (6,400 AU/ml) in the absence of glycerol (Fig. 4d). Glycerol concentrations of 0.2% (w/v) and higher (up

to 5.0%, w/v) repressed bacteriocin production progressively (Fig. 4d). Similar results were reported for the production of plantaricin ST31, with concentrations higher than 0.2% leading to a decrease in activity (Todorov et al., 2000).

The addition of cyanocobalamin and thiamine in MRS broth (1.0 ppm) yielded 1,600 AU/ml bacteriocin, whereas L-ascorbic acid and DL-6,8-thioctic acid resulted in 200 AU/ml. Vitamin K₁ stimulated bacteriocin ST13BR production (3,200 AU/ml). Maximal bacteriocin activity (6,400 AU/ml) was recorded in MRS without added vitamins (Fig. 4d).

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