Journal of Applied Microbiology 1999, **87**, 726–734

Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus pentosus* with a fungistatic effect on *Candida albicans*

D.J. Okkers[1], L.M.T. Dicks[1], M. Silvester[1], J.J. Joubert[2] and H.J. Odendaal[3]

[1]Department of Microbiology, [2]Department of Medical Microbiology and [3]Department of Gynaecology and Obstetrics, University of Stellenbosch, South Africa

7192/04/99: received 26 April 1999, revised 2 July 1999 and accepted 8 July 1999

D.J. OKKERS, L.M.T. DICKS, M. SILVESTER, J.J. JOUBERT AND H.J. ODENDAAL. 1999. *Lactobacillus pentosus* TV35b, isolated from the posterior fornix secretions of the vagina of a prenatal patient, produced a bacteriocin-like peptide (pentocin TV35b), which is inhibitory to *Clostridium sporogenes*, *Cl. tyrobutyricum*, *Lact. curvatus*, *Lact. fermentum*, *Lact. sake*, *Listeria innocua*, *Propionibacterium acidipropionici*, *Propionibacterium* sp. and *Candida albicans*. The mechanism of activity of pentocin TV35b is bactericidal, as shown by a decrease in the viable cell numbers of *Lact. sake* from approximately 4×10^8 to less than 10 cfu ml^{-1} over a period of 4 h. Pentocin TV35b added to the growth medium of *C. albicans* stimulated the formation of pseudohyphae during the first 36 h, followed by a slight repression in cell growth. Production of pentocin TV35b was at its maximum towards the end of the logarithmic growth phase of strain TV35b. The peptide was purified by ammonium sulphate precipitation, followed by SP-Sepharose cation exchange chromatography. The molecular size of pentocin TV35b was estimated to be between 2·35 and 3·4 kDa, according to tricine-SDS PAGE. However, results obtained by electrospray ionization mass spectroscopy indicated that the peptide is 3930·2 Da in size. Amino acid analysis performed by using the Pico-Tag® method and a Nova-Pak C_{18} HPLC column indicated that pentocin TV35b consists of 33 amino acids with a total mass of 3929·63 Da. Pentocin TV35b is inactivated when treated with papain and Proteinase K, but remains active after incubation at pH 1–10 for 2 h at 25 °C, and when heat-treated for 30 min at 100 °C.

INTRODUCTION

Women world-wide have been plagued by vaginal infections caused by *Candida albicans*, *Gardnerella vaginalis* and *Trichomonas* spp., generally referred to as candidiasis, bacterial vaginosis (BV) and trichomoniasis, respectively (Sobel *et al.* 1984; Friedrich 1985). If the vaginal pH during pregnancy increases above 4·4, infections by *Trichomonas vaginalis*, *Bacteroides* spp. and *Mycoplasma hominis* may occur, leading to upper genital tract and intra-amniotic infections (Newton *et al.* 1996; Peipert *et al.* 1997). This could lead to serious complications, e.g. pre-term birth, low-birth-weight infants, premature rupture of membranes, chorioamnionitis, and postcaesarean and postpartum endometritis (Chaim *et al.* 1997; Ferris 1998; Paige *et al.* 1998).

Correspondence to: Prof. L.M.T. Dicks, Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa (e-mail: lmtd@maties.sun.ac.za).

Lactobacillus acidophilus, *Lact. plantarum*, *Lact. brevis*, *Lact. fermentum*, and to a lesser extent, *Lact jensenii* and *Lact. casei* have been isolated from the human vagina (Fernandes *et al.* 1987). It is generally assumed that the vaginal lactobacilli are responsible for protection against pathogenic micro-organisms (Collins and Hardt 1980; Redondo-Lopez *et al.* 1990; McGroarty 1993; Mital and Garg 1995). Women colonized with lactic acid bacteria are less likely to develop BV and symptomatic candidiasis, and do not have *G. vaginalis*, *Bacteroides* spp., *Peptostreptococcus* spp., *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Streptococcus viridans*. *Lactobacillus acidophilus* inhibits the growth of *G. vaginalis* and *Bacteroides bivius* (Hillier *et al.* 1992; Larsen 1993).

In this paper we report on a bacteriocin-like peptide with

anti-*Candida* activity, produced by a strain of *Lactobacillus pentosus* which has been isolated from the posterior fornix secretions of the human vagina.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus pentosus TV35b was isolated from the posterior fornix vaginal secretions of a prenatal patient at Tygerberg Hospital, Tygerberg, South Africa. The indicator strains used in this study are listed in Table 1. The bacterial reference strains were from the Laboratorium voor Microbiologie, University of Ghent (LMG), Ghent, Belgium, the American Type Culture Collection (ATCC) and the National Collection of Industrial and Marine Bacteria (NCIMB). The *Candida albicans* strains were isolated from patients during routine examinations in the Department of Medical Microbiology (University of Stellenbosch, Tygerberg, South Africa). The bacteria were maintained in the growth media recommended in the respective culture collection catalogues. The *C. albicans* strains were grown in YPD medium (yeast extract 10 g l^{-1}, peptone 20 g l^{-1}, glucose 20 g l^{-1}).

Table 1 Spectrum of antimicrobial activity of pentocin TV35b

Organism	Strain	Activity
Bacillus cereus	LMG 13569	−
Clostridium sporogenes	LMG 13570	++
Clostridium tyrobutyricum	LMG 13571	++
Enterococcus faecalis	LMG 13566	−
Gardnerella vaginalis	ATCC 14019	−
Lactobacillus acidophilus	LMG 13550	−
Lactobacillus casei	LMG 13552	−
Lactobacillus curvatus	LMG 13553	+
Lactobacillus fermentum	LMG 13554	++
Lactobacillus helveticus	LMG 13555	−
Lactobacillus plantarum	LMG 13556	−
Lactobacillus reuteri	LMG 13557	−
Lactobacillus sake	LMG 13558	++
Lactobacillus vaginalis	ATCC 49540	−
Leuconostoc cremoris	LMG 13562	−
	LMG 13563	−
Listeria innocua	LMG 13568	++
Pediococcus pentosaceus	LMG 13560,	−
	LMG 13562	−
Peptostreptococcus vaginalis	ATCC 51170	−
Propionibacterium acidipropionici	LMG 13572	+++
Propionibacterium sp.	LMG 13573,	+++
	LMG 13574	+++
Proteus mirabilis	ATCC 25933	−
Staphylococcus carnosus	LMG 13567	−
Streptococcus thermophilus	LMG 13564,	−
	LMG 13565	−
Candida albicans	MCB1, MCB2,	++
	MCB3, MCB4	++

+, Inhibition zone 5–10 mm; ++, inhibition zone 10–15 mm; +++, inhibition zone > 15 mm.
−, Resistant to the bacteriocin.

Identification of strain TV35b

Strain TV35b was identified by numerical analyses of total soluble cell protein patterns and RAPD-PCR profiles according to the methods described by Vauterin *et al.* (1993) and Van Reenen and Dicks (1996), respectively. Sequencing of the 16S rRNA of strain TV35b was performed as described by Falsen *et al.* (1999).

Inhibitory activity

Lactobacillus pentosus TV35b was inoculated (1% v/v) into MRS broth (Biolab) and incubated at 30 °C without aeration until mid-logarithmic phase of growth (O.D. 600 = 1·4). The spot-on-lawn method described by Mayr-Harting *et al.* (1972) was used to determine the antimicrobial activity of pentocin TV35b against the organisms listed in Table 1. An aliquot of 10 μl cell-free culture supernatant fluid, adjusted to pH 6·5 with 0·1 N NaOH, was spotted onto an agar plate (0·7% w/v agar) seeded with active growing cells of the test organism (approximately 10^6 cells ml^{-1}). Plates were incubated at the optimal growth temperature of the test organism, as indicated in the respective culture collection catalogues. A clear zone of inhibition of at least 2 mm in diameter was recorded as positive.

Production of pentocin TV35

Production of pentocin TV35b was followed during growth of *Lact. pentosus* TV35b in MRS broth (Biolab). At specific time intervals, samples of 1 ml were removed, the optical density (at 600 nm) of the culture determined and the arbitrary activity units (AU) per millilitre (reciprocal of the highest dilution at which activity was still obtained) of pentocin TV35b was determined according to the method of Henderson *et al.* (1992). *Lactobacillus sake* LMG 13558 was used as indicator strain.

Isolation and purification of pentocin TV35b

Lactobacillus pentosus TV35b was grown in MRS broth (Biolab) at 37 °C until mid-logarithmic phase ($O.D._{600}$ = 1·4). The cells were harvested and pentocin TV35b was isolated from the cell-free supernatant fluid by ammonium sulphate (55% w/v final concentration) pre-

cipitation, as described by Bollag and Edelstein (1991). The precipitate was recovered by centrifugation (15 min at 11 300 *g*), resuspended in sterile MilliQ water and dialysed against sterile MilliQ water at 8 °C for 24 h, using a 1 kDa cut-off dialysis membrane (Spectrum). The pH of the desalted sample was adjusted to approximately 6·5 with 0·1 N NaOH and freeze dried.

Further purification of pentocin TV35b was performed by cation-exchange chromatography using a SP-Sepharose Fast Flow (Pharmacia-LKB) column. Crude pentocin TV35b (6 ml; 9 mg protein ml^{-1}), suspended in 0·05 mol l^{-1} sodium acetate buffer (pH 6), was applied onto 8 ml SP-Sepharose Fast Flow matrix, which had been equilibrated with the same buffer. The column was washed with 20 ml acetate buffer and the proteins eluted with an NaCl step-gradient of 0·1–1·0 mol l^{-1} (in acetate buffer, pH 6), at 2 ml min^{-1}. Fractions of 1 ml were collected and tested for pentocin TV35 activity against *Lact. sake* LMG 13558, as described previously. The active fractions were pooled, dialysed against sterile MilliQ H_2O and divided into three equal parts of approximately 2 ml and freeze dried. The freeze-dried powder was resuspended in 100 μl sterile MilliQ H_2O and tested for activity against *Lact. sake* LMG 13558 and *Cand. albicans* MCB 1.

Sensitivity to heat, pH and proteolytic enzymes

Crude extract samples of pentocin TV35b (approximately 9 mg protein ml^{-1} after ammonium sulphate precipitation) were used in these tests. *Lactobacillus sake* LMG 13558 was used as indicator organism. Aliquots of pentocin TV35b were exposed to heat treatments of 60, 80 and 100 °C for 10, 20 and 30 min, respectively, and 121 °C for 15 min, followed by immediate cooling on ice and testing for antimicrobial activity, as described before. In a separate experiment, samples of pentocin TV35b were adjusted to pH values ranging from 1 to 10, incubated at 25 °C for 2 h, neutralized to pH 7, and tested for bactericidal activity. Resistance of pentocin TV35b to proteolytic enzymes was determined by incubation of the bacteriocin samples in the presence of Proteinase K (10 U mg^{-1} pentocin TV35b), pepsin (1250 U mg^{-1} pentocin TV35b), papain (15 U mg^{-1} pentocin TV35b), α-chymotrypsin (45 U mg^{-1} pentocin TV35b) and trypsin (55 U mg^{-1} pentocin TV35b) at 37 °C for 2 h. After incubation the enzymes were heat-inactivated (3 min at 100 °C) and the incubate tested for antimicrobial activity.

Mode of action

Partially-purified pentocin TV35b (128 AU ml^{-1}) was added to mid-logarithmic growth phase cells (approximately 4×10^8 cfu ml^{-1}) of *Lact. sake* LMG 13558 in 200 ml MRS broth (Biolab) and lag-phase cells of *C. albicans* MCB1 (approximately 1×10^8 cfu ml^{-1}) in 200 ml YPD medium. In both experiments, concentrated medium (MRS or YPD) was added to control flasks. Changes in the turbidity of the cultures were recorded at an O.D. of 600 nm and the number of colony-forming units (cfu) was determined by plating the samples on MRS and YPD agar. Gram stains of the *C. albicans* cells were made after 8 and 24 h of growth in the presence and absence of pentocin TV35b.

Size determination

Samples collected from the SP-Sepharose column were separated on a tricine-SDS-polyacrylamide gel (Schägger and von Jagow 1987). A low molecular weight protein marker with sizes ranging from 2·35 to 46 kDa (Amersham International, UK) was used. The gels were fixed and one half was stained with Coomassie Brilliant Blue R250 (Saarchem, Krugersdorp, South Africa). The other half of the gel was pre-washed with sterile distilled water and overlayed with active growing cells of *Lact. sake* LMG13558 (approximately 10^6 ml^{-1}), embedded in MRS agar (0·7% w/v). After 2 days of incubation at 30 °C, the formation of an inhibition zone indicated the position and size of the active pentocin TV35b in the gel.

Confirmation of the molecular size of pentocin TV35b was obtained by analysing the purified peptide in a Quattro triple quadropole mass spectrometer (Micromass, Manchester, UK). The sample was diluted in 10/90 acetonitrile/water containing 0·01% formic acid. A 10 μl sample of this dilution was injected via a Rheodyne injection port. The carrier solvent was 50/50 acetonitrile/water at a flow rate of 20 μl min^{-1}, delivered by a Pharmacia-LKB 2249 HPLC pump. The source temperature was 80 °C and the capillary voltage, 3·5 kV with the cone voltage at 60 V. All other lenses were set for maximum detection of the compounds. Data were acquired in the continuum mode and the m/z scale scan from 400 to 2000 at 5 s per scan. Scans across the elution peak were combined, the background subtracted and the multiple charged spectrum calculated using the MaxEnt program.

Amino acid analysis

Amino acid analysis of pentocin TV35b was done on samples collected from the SP-Sepharose column according to the Pico-Tag® method (Bidlingmeyer *et al.* 1984). The sampled fractions were vacuum-dried and then subjected to 1 h hydrolysis at 150 °C in the presence of N_2 and 6 N HCl containing 1% phenol. The acid was removed from the samples under high vacuum, followed by the addition of 10 μl methanol : water : tetraethylammonium (TEA) (1 : 2 : 1), and again vacuum-dried. Pre-column derivatization was done with 20 μl methanol : TEA: water : phenylisothiocyanate (PITC) (7 : 1 : 1 : 1) for 20 min at room temperature. The samples were vacuum-dried, dissolved in an appropriate amount (50–300 μl) of Pico-Tag® diluent and filtered through HV 0·45 μm Millipore filters.

Chromatography was done at 44 °C, using a Nova-Pak C_{18} HPLC column (150 mm × 3·9 mm), on a Waters HPLC system consisting of two Waters 510 pumps, a MAXIMA controller system, a Waters model 440 detector and a WISP 712 sample processor. Separation of the derivatized amino acids was accomplished with a binary non-linear gradient using eluant A (0·14 mol l^{-1} sodium acetate, 10 mmol l^{-1} EDTA and 0·5 ml TEA l^{-1}, titrated to pH 6·40 with acetic acid mixed with 6% acetonitrile) and eluant B (60% acetonitrile containing 10 mmol l^{-1} EDTA). A Pierce standard amino acid mixture (hydrolysis standard) was used to calibrate the analyses.

RESULTS

Strain TV35b clustered with four other strains isolated from the posterior fornix secretions of the vagina, and *Lact. pentosus* NCIMB 8531, in one protein profile cluster at $r \geqslant 0{\cdot}82$ (Fig. 1). The DNA profile of strain TV35b was almost identical to that of the type strain of *Lact. pentosus* (NCIMB 8026^T), as indicated by their close grouping at $R^2 \geqslant 0{\cdot}96$ (Fig. 2). The 16S rRNA sequence of strain TV35b was identical to that of *Lact. pentosus* NCIMB 8026^T (data not shown), confirming its classification as *Lact. pentosus.*

Pentocin TV35b was active against *Clostridium sporogenes*, *Cl. tyrobutyricum*, *Lact. curvatus*, *Lact. fermentum*, *Lact. sake*, *Listeria innocua*, *Propionibacterium acidipropionici*, *Propionibacterium* sp. and *C. albicans* (Table 1). The addition of pentocin TV35b to cells of *Lact. sake* LMG 13558 in their mid-logarithmic growth phase resulted in a rapid decrease in the number of viable cells (approximately 4×10^8 to less than 10 cfu ml^{-1}) over a period of 4 h (Fig. 3). The optical density readings of *Lact. sake* LMG 13558 declined from 1·75 to 1·65 4 h after pentocin TV35b was added and then stabilized at O.D. 1·65 for the duration of the incubation period (Fig. 3).

The optical density readings of *C. albicans* MCB1 were very similar in the presence and absence of pentocin TV35b (Fig. 4). However, cells grown in the presence of pentocin TV35b developed pseudohyphae (Fig. 5) during the first 36 h of incubation, followed by a slow increase in cell count ($1{\cdot}5 \times 10^8$–2×10^8 cfu ml^{-1}) over 12 h (Fig. 4). The number of viable cells grown in the absence of pentocin TV35b increased from 1×10^8–8×10^8 during the same period (Fig. 4).

Production of pentocin TV35 started during early exponent-

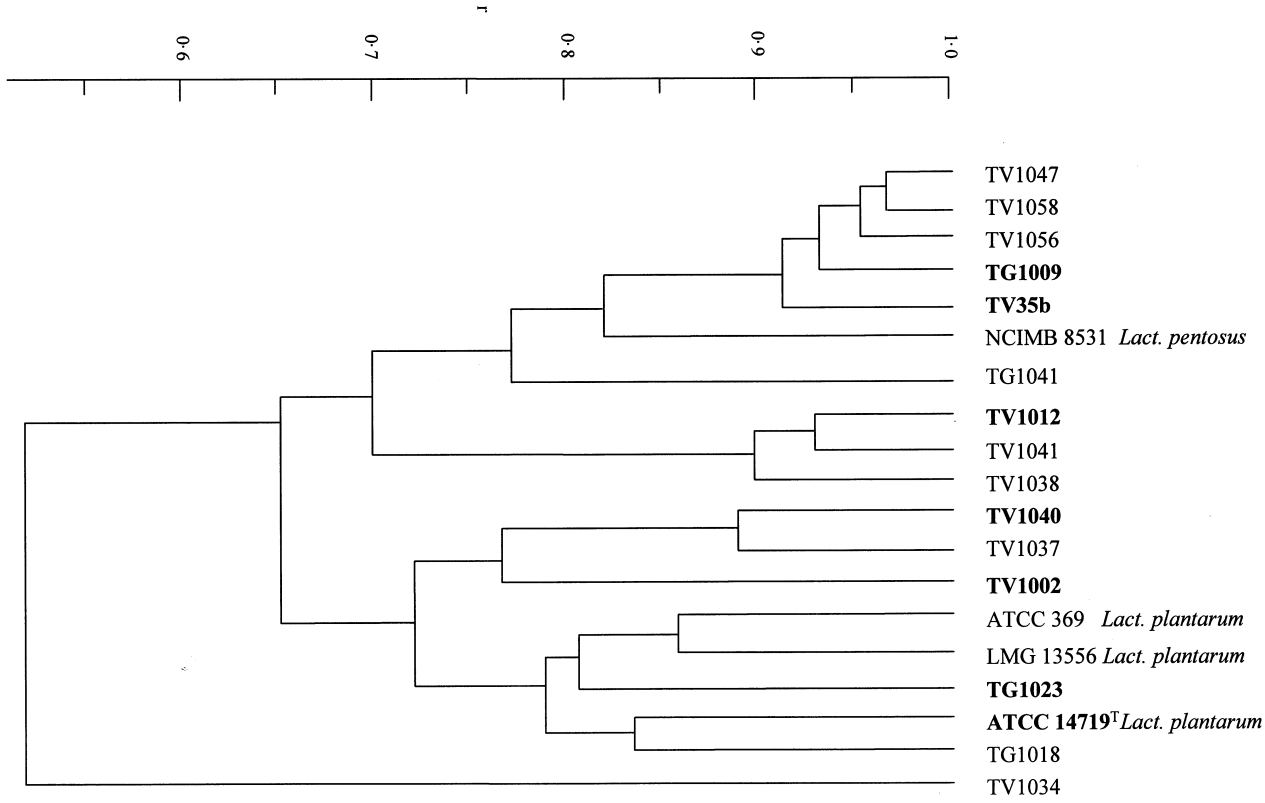

Fig. 1 Simplified dendrogram showing the clustering of strain TV35b with *Lactobacillus pentosus* and *Lact. plantarum* obtained by numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA. Strains printed in bold were selected for RAPD-PCR analysis

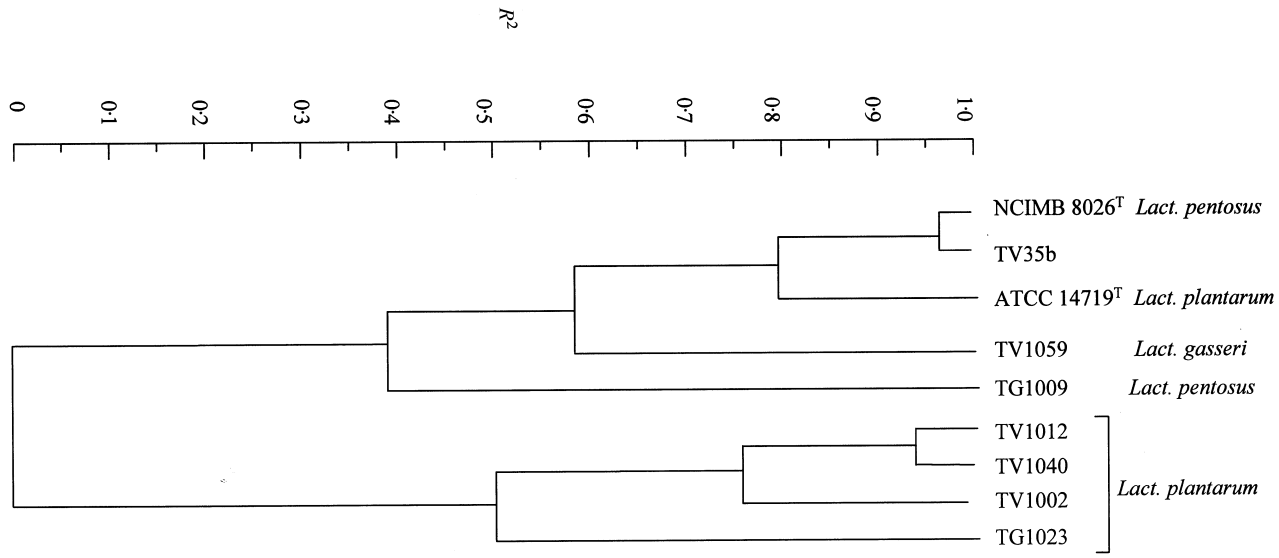

Fig. 2 Dendrogram showing the clustering of strain TV35b with *Lactobacillus pentosus*, *Lact. plantarum* and *Lact. gasseri* obtained by numerical analysis of RAPD-PCR profiles. Clustering was by the normalized average linkage analysis. Distances between clusters are expressed in R^2-values

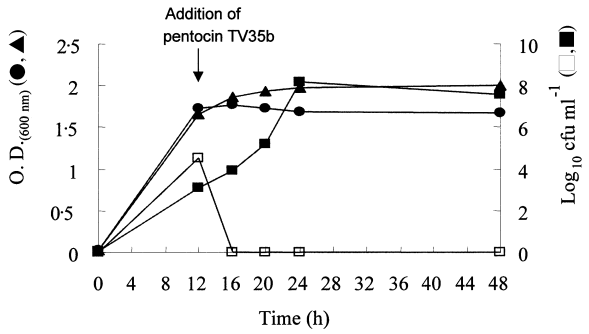

Fig. 3 The effect of pentocin TV35b on the growth of *Lactobacillus sake* LMG 13558. (▲) and (●), turbidity of the cells (measured at O.D. of 600 nm) growing in the absence and presence of pentocin TV35b, respectively. (■) and (□), cfu ml^{-1} counts in the absence and presence of pentocin TV35b, respectively

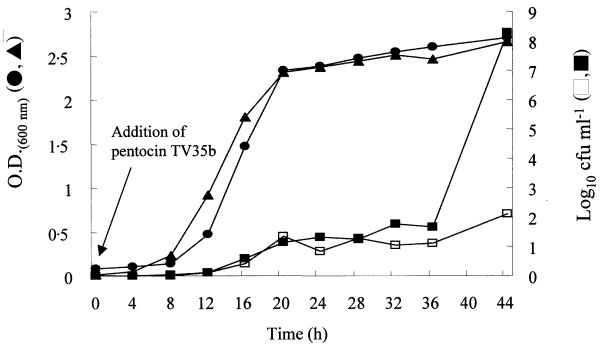

Fig. 4 The effect of pentocin TV35b on the growth of *Candida albicans* MCB1. (▲) and (●), turbidity of the cells (measured at O.D. of 600 nm) growing in the absence and presence of pentocin TV35b, respectively. (■) and (□), cfu ml^{-1} counts in the absence and presence of pentocin TV35b, respectively

ial growth and more or less followed the trend of the growth curve. After a short lag phase of approximately 4 h, the cells of *Lact. pentosus* TV35b increased to a maximum optical density (O.D. 600 nm) of 2·50 (Fig. 6). Maximum pentocin TV35b production (3250 AU ml^{-1}) was recorded after 12 h of growth, i.e. towards late logarithmic growth phase (Fig. 6). Pentocin TV35b activity remained at 3250 AU ml^{-1} for 21 h, after which it decreased to 1600 AU ml^{-1} for the duration of the incubation period (Fig. 6). The pH declined from 5·8 to 3·0 during the 48 h incubation period.

Isolation of pentocin TV35b with ammonium sulphate precipitation, followed by cation exchange chromatography with SP-Sepharose and separation on a tricine-SDS-PAGE gel, yielded an active peptide band corresponding in size to 2·35–3·4 kDa (Fig. 7). Analysis of the purified peptide by electrospray ionization mass spectroscopy revealed a molecular size of 3930·2 Da (Fig. 8).

The probable amino acid composition of pentocin TV35b, as determined by the Pico-Tag® method and separation using a Nova-Pak C_{18} HPLC column, is shown in Table 2. Based on these results, pentocin TV35b consists of 33 amino acids with a total molecular mass of 3929·63 Da, corresponding to the molecular size determined by electrospray ionization mass spectroscopy.

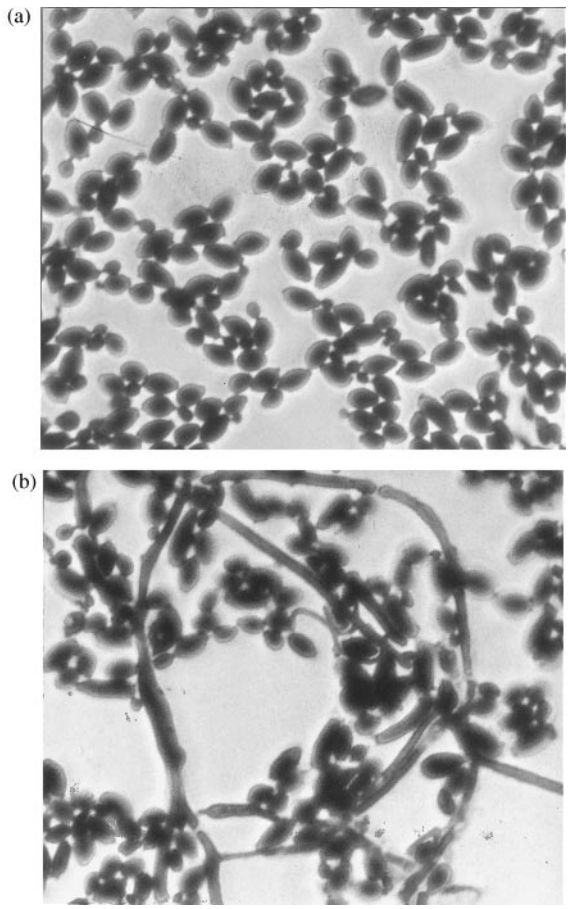

Fig. 5 The effect of pentocin TV35b on the morphology of *Candida albicans* MCB1. (a) Growth in the absence of pentocin TV35b; (b) growth in the presence of pentocin TV35b

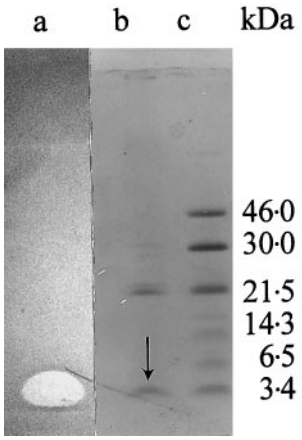

Fig. 7 Separation of pentocin TV35b by tricine-SDS-PAGE. (a) Gel overlaid with cells of *Lactobacillus sake* LMG 13558 embedded in MRS Agar (0·7% (w/v) agar); (b) partially purified crude extract and (c) Rainbow protein molecular weight marker. The active protein band is indicated by the arrow

Pentocin TV35b was resistant to heat treatments of 60, 80 and 100 °C for 30 min, but lost 50% of its activity after 15 min at 121 °C. Incubation in buffers at pH values ranging from 1 to 10 had no effect on the activity of pentocin TV35b. The peptide was resistant to treatment with catalase, pronase, trypsin, lysozyme and lipase, but was inactivated by papain and Proteinase K. No rest–activity was obtained after treatment with these proteolytic enzymes.

DISCUSSION

Lactobacillus pentosus and *Lact. plantarum* are phenotypically closely related and difficult to differentiate based on simple physiological tests (Dellaglio *et al.* 1975; Kandler and Weiss 1986), including total soluble cell protein patterns (Van Reenen and Dicks 1996). However, numerical analysis of RAPD-PCR proved a reliable technique for differentiating *Lact. pentosus* from *Lact. plantarum* (Van Reenen and Dicks 1996). Based on our results, strain TV35b is phenotypically

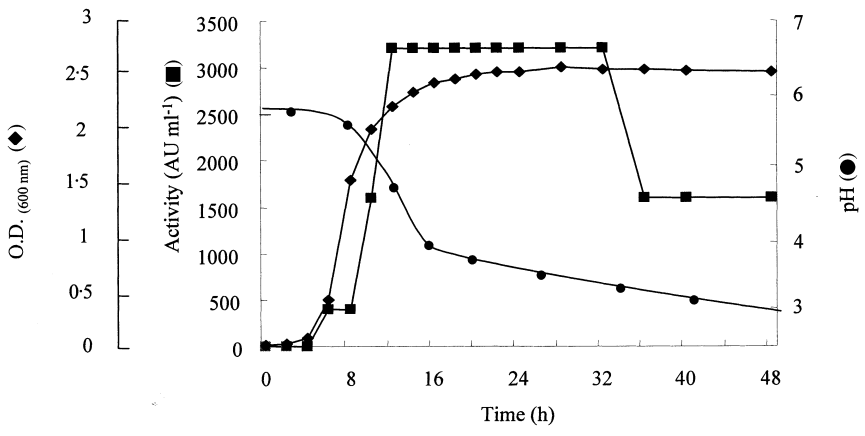

Fig. 6 Production of pentocin TV35b during the growth of *Lactobacillus pentosus* TV35b. (◆), Optical density at 600 nm; (■), pentocin TV35b activity in AU ml^{-1}; (●), changes in pH

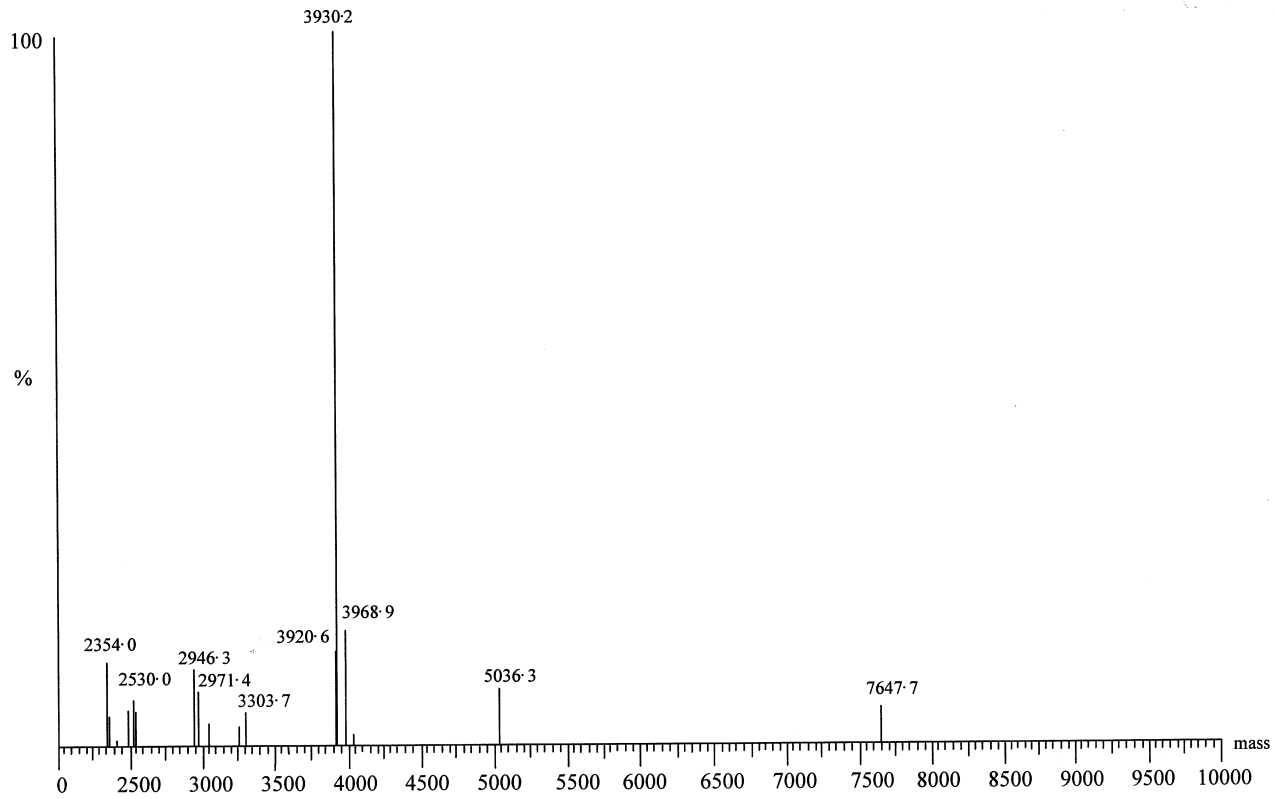

Fig. 8 Electrospray mass spectroscopy of pentocin TV35b, obtained by a Quattro triple quadropole mass spectrometer. Data were acquired in the continuum mode and the m/z scale scan from 400 to 2000 at 5 s scan^{-1}

(Fig. 1) and genetically (Fig. 2) related to *Lact. pentosus*. Sequencing of the 16S rRNA of strain TV35b confirmed its classification as *Lact. pentosus*.

The inhibition of *Listeria innocua* classifies pentocin TV35b as a member of the group IIa (anti-*Listeria*) bacteriocins, according to the classification system proposed by Klaenhammer (1993). However, pentocin TV35b is not antimicrobial towards *Bacillus cereus*, *Enterococcus faecalis*, *Lact. helveticus*, *Lact. plantarum*, *Pediococcus pentosaceus*, *Staphylococcus carnosus* and *Streptococcus thermophilus* and is in this respect, is different from many anti-*Listeria* bacteriocins, including most plantaricins (Van Reenen *et al.* 1998). Furthermore, the antifungal activity of pentocin TV35b, as observed against *C. albicans* MCB1, is contradictory to the generally accepted definition of bacteriocins (Klaenhammer 1993), and the peptide cannot therefore be defined as a true bacteriocin. *Lactobacillus pentosus* TV35b has been isolated from the human vagina among strains of *C. albicans*. It might therefore be likely that strain TV35b acquired its anti-*Candida* characteristic through natural selection in this highly specialized niche. Further studies are needed to substantiate this hypothesis.

The rapid decline in number of living cells of *Lact. sake* LMG 13558 over a period of 4 h after the addition of pentocin TV35b (4×10^8 to less than 10 cfu ml^{-1}) suggests that the mode of activity of pentocin TV35b is bactericidal (Fig. 3). However, little variation was recorded in the optical density (O.D. 600) values of the cells of *Lact. sake* LMG 13558, indicating that they were not lysed (Fig. 3). The formation of pseudohyphae by cells of *C. albicans* MCB1 grown in the presence of pentocin TV35b (Fig. 5) is an indication that the cells are under stress, as is also evident from the slow increase in cell numbers after 36 h (Fig. 4). This is the first report of an anti-*Candida* peptide being produced by a *Lactobacillus* sp. Further research is needed to determine the mechanism of the antifungal activity.

Production of pentocin TV35b more or less followed the growth curve of the producer organism, with maximum levels of activity (3250 AU ml^{-1}) after 12 h of growth and a pH of 4·5. This level of activity was maintained for 21 h, after which it declined to 1600 AU ml^{-1} for the remaining period of fermentation (Fig. 6). Similar results were obtained for nisin (Hurst and Dring 1968), pediocin AcH (Yang *et al.* 1992) and plantaricin 423 (Verellen *et al.* 1998). In the case of nisin,

Table 2 Probable amino acid composition of pentocin TV35b

Amino acid	No. of residues	Mr of amino acid	Molecular mass
Alanine	1	71·09	71·09
Arginine	2	156·19	312·38
Aspartic acid	2	115·10	230·20
Cysteine	1	103·14	103·14
Glutamic acid/ Glutamine	3	129·13	387·39
Glycine	1	57·05	57·05
Histidine	3	137·14	411·42
Isoleucine	1	113·16	113·16
Leucine	3	113·16	339·48
Lysine	2	128·17	256·34
Methionine	1	131·20	131·20
Phenylalanine	2	147·18	294·36
Proline	3	97·12	291·36
Serine	1	87·09	87·09
Threonine	2	101·11	202·22
Tyrosine	2	163·18	326·36
Valine	3	99·13	297·39
Total	33		3929·63

more than 80% of the peptide remained adsorbed to the producer cell (*Lactococcus lactis* subsp. *lactis*) at a pH of 6·8. However, with a decrease in pH to below 6·0, more than 80% of the nisin was released from the surface of the cell wall (Hurst and Dring 1968). In the case of pediocin AcH, prepediocin modifying enzymes are activated at a pH below 5·0, which converts the inactive prepediocin AcH into its active form (Ray 1994). In the case of plantaricin 423, produced by *Lact. plantarum* 423, a higher level of the bacteriocin is produced in medium with an initial pH of 4·9 (Verellen *et al.* 1998). It may therefore be that the production of pentocin TV35b is pH controlled with maximum production at a pH below 4·5. Further research is needed to determine if prepentocin enzymes are involved in the activation of pentocin TV35b.

Pentocin TV35b is estimated to be approximately 3930 Da in size, based on results obtained from electrospray ionization mass spectroscopy (Fig. 8). The molecular size was confirmed by amino acid analysis (Table 2). It is concluded from the amino acid analysis that pentocin TV35b is not that different from bacteriocins described for other *Lactobacillus* spp., including *Lact. plantarum* (De Vuyst and Vandamme 1994).

The heat stability of pentocin TV35b (up to 30 min at 100 °C) is similar to that reported for the bacteriocin plantaricin 423 (Van Reenen *et al.* 1998), plantaricin A (Daeschel *et al.* 1990), plantaricin C19 (Atrih *et al.* 1993), plantaricin S (Jiménez-Díaz *et al.* 1993), plantaricin 149 (Kato *et al.* 1994) and plantaricin SA6 (Rekhif *et al.* 1995).

ACKNOWLEDGEMENTS

Data on the 16S rRNA sequencing were kindly provided by Dr M.D. Collins, Department of Food Science and Technology, University of Reading, Reading Laboratory, Reading RG6 6AP, UK. Amino acid analysis and mass spectroscopy were performed by Dr M. Rautenbach and Dr T. van der Merwe, respectively, Department of Biochemistry, University of Stellebosch, Stellenbosch 7600, South Africa. The project was funded by the Industrial Development Corporation, South Africa.

REFERENCES

Atrih, A., Rekhif, N., Milliere, J.B. and Levebvre, G. (1993) Detection and characterization of a bacteriocin produced by *Lactobacillus plantarum* C19. *Canadian Journal of Microbiology* **39,** 1173–1179.

Bidlingmeyer, B.A., Cohen, S.A. and Tarvin, T.L. (1984) Rapid analysis of amino acids using pre-column derivitization. *Journal of Chromatography* **336,** 93–104.

Bollag, D.M. and Edelstein, S.J. (1991) Concentrating protein solutions. In *Protein Methods.* pp. 71–79. New York, NY: Wiley-Liss Inc.

Chaim, W., Mazor, M. and Leiberman, J.R. (1997) The relationship between bacterial vaginosis and preterm birth. A review. *Archives of Gynecology and Obstetrics* **259,** 51–58.

Collins, E.B. and Hardt, P. (1980) Inhibition of *Candida albicans* by *Lactobacillus acidophilus*. *Journal of Dairy Science* **63,** 830–832.

Daeschel, M.A., McKenney, M.C. and McDonald, L.C. (1990) Bacteriocidal activity of *Lactobacillus plantarum* C-11. *Food Microbiology* **7,** 91–98.

De Vuyst, L. and Vandamme, E.J. (1994) Antimicrobial potential of lactic acid bacteria. In *Bacteriocins of Lactic Acid Bacteria, Microbiology, Genetics and Applications* ed. De Vuyst, L. and Vandamme, E.J. pp. 91–141. London: Blackie Academic and Professional.

Dellaglio, F., Bottazzi, V. and Vescovo, M. (1975) Deoxyribonucleic acid homology among *Lactobacillus* species of the subgenus *Streptobacterium* Orla-Jensen. *International Journal of Systematic Bacteriology* **25,** 160–172.

Falsen, E., Pascual, C., Sjöden, B., Ohlén, M. and Collins, M.D. (1999) Phenotypic and phylogenetic characterization of a novel *Lactobacillus* species from human sources: description of *Lactobacillus iners* sp. nov. *International Journal of Systematic Bacteriology* **49,** 217–221.

Fernandes, C.F., Shahani, K.M. and Amer, M.A. (1987) Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy products. *FEMS Microbiology Reviews* **46,** 343–356.

Ferris, D.G. (1998) Management of bacterial vaginosis during pregnancy. *American Family Physician* **57,** 1215–1228.

Friedrich, E.G. (1985) Vaginitis. *American Journal of Obstetrics and Gynecology* **152,** 247–251.

Henderson, J.T., Chopko, A.L. and van Wassenaar, P.D. (1992) Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC1.0. *Archives of Biochemistry and Biophysics* **295,** 5–12.

Hillier, S.L., Krohn, M.A., Klebanoff, S.J. and Eschenbach, D.A. (1992) The relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women. *Obstetrics and Gynecology* **79,** 369–373.

Hurst, A. and Dring, G.J. (1968) The relation of the length of lag phase growth to the synthesis of nisin and other basic proteins by *Streptococcus lactis* grown under different cultural conditions. *Journal of General Microbiology* **50,** 383–390.

Jiménez-Díaz, R., Rios-Sánchez, R.M., Desmazeaud, M., Ruiz-Barba, J.L. and Piard, J.-C. (1993) Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. *Applied and Environmental Microbiology* **59,** 1416–1424.

Kandler, O. and Weiss, N. (1986) Genus *Lactobacillus* Beijerinck 1901, 212AL. In *Bergey's Manual of Determinative Bacteriology,* Vol. 2, ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. pp. 1209–1234. Baltimore: The Williams & Wilkins Co.

Kato, T., Matsuda, T., Ogawa, E. *et al.* (1994) Plantaricin 149, a bacteriocin produced by *Lactobacillus plantarum* NRIC 149. *Journal of Fermentation and Bioengineering* **77,** 277–282.

Klaenhammer, T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews* **12,** 39–86.

Larsen, B. (1993) Vaginal flora in health and disease. *Clinical Obstetrics and Gynecology* **36,** 107–121.

Mayr-Harting, A., Hedges, A.J. and Berkeley, R.C.W. (1972) Methods for studying bacteriocins. *Methods in Microbiology* **12,** 39–86.

McGroarty, J.A. (1993) Probiotic use of lactobacilli in the human female urogenital tract. *FEMS Immunology and Medical Microbiology* **6,** 251–264.

Mital, B.K. and Garg, S.K. (1995) Anticarcinogenic, hypo-cholesterolemic, and antagonistic activities of *Lactobacillus acidophilus*. *Critical Reviews in Microbiology* **21,** 175–214.

Newton, E.R., Piper, J. and Peairs, W. (1996) Bacterial vaginosis and intraamniotic infection. *American Journal of Obstetrics and Gynecology* **176,** 672–677.

Paige, D.M., Augustyn, M., Adih, W.K., Witter, F. and Chang, J. (1998) Bacterial vaginosis and preterm birth: a comprehensive review of the literature. *Journal of Nurse-Midwifery* **43,** 83–89.

Peipert, J.F., Montagno, A.B., Cooper, A.S. and Sung, C.J. (1997) Bacterial vaginosis as a risk factor for upper genital tract infection. *American Journal of Obstetrics and Gynecology* **177,** 1184–1187.

Ray, B. (1994) Pediocins of *Pediococcus* species. In *Bacteriocins of Lactic Acid Bacteria, Microbiology, Genetics and Applications*, ed. De Vuyst, L. and Vandamme, E.J. pp. 465–495. London: Blackie Academic and Professional.

Redondo-Lopez, V., Cook, R.L. and Sobel, J.D. (1990) Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. *Reviews of Infectious Diseases* **12,** 856–872.

Rekhif, N., Atrih, A. and Lefebvre, G. (1995) Activity of plantaricin SA6, a bacteriocin produced by *Lactobacillus plantarum* SA6 isolated from fermented sausage. *Journal of Applied Bacteriology* **78,** 349–358.

Schägger, H. and Von Jagow, G. (1987) Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1–100 kDa. *Analytical Biochemistry* **166,** 368–379.

Sobel, J.D., Muller, G. and Buckley, H.R. (1984) Critical role of germ tube formation in the pathogenesis of candidal vaginitis. *Infection and Immunity* **44,** 576–580.

Van Reenen, C.A. and Dicks, L.M.T. (1996) Evaluation of numerical analysis of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* and *Lactobacillus pentosus*. *Current Microbiology* **32,** 183–187.

Van Reenen, C.A., Dicks, L.M.T., Chikindas, M., Verellen, T.L.J. and Vandamme, E.J. (1998) Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology* **84,** 1131–1137.

Vauterin, L., Swings, J. and Kersters, K. (1993) Protein electrophoresis and classification. In *Handbook of New Bacterial Systematics* ed. Goodfellow, M. and O'Donnell, A.G. pp. 251–280. London: Academic Press.

Verellen, T.L.J., Bruggeman, G., Van Reenen, C.A., Dicks, L.M.T. and Vandamme, E.J. (1998) Fermentation optimization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Fermentation and Bioengineering* **86,** 174–179.

Yang, R., Johnson, M.C. and Ray, B. (1992) Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology* **58,** 3355–3359.