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Fermenting cucumber, a potential source for the isolation of pediocin-like bacteriocin producers

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Summary

A strain of *Pediococcus acidilactici* CFR K7 isolated from cucumber, produced an antimicrobial peptide which acted against *Leuconostoc mesenteroides*, selected strains of *Lactobacillus* spp., *Pediococcus* spp. and *Enterococcus* spp. The partially purified bacteriocin had molecular weight of ~4.6 kDa, heat stability in a range of 40–121 °C and was active over a wide range of pH (2.0–9.0). This bacteriocin possessed strong antilisterial activity and was susceptible to proteolytic enzymes. Southern hybridization using the PCR-generated *ped*A probe established that the gene for the bacteriocin was plasmid-borne as in the case of pediocin PA-1. Nucleotide sequence of the *ped*AB gene indicated 100% homology to a pediocin AcH/PA-1. Certain bacteriocinogenic strains isolated from naturally fermented cucumber were tested by colony hybridization using the *ped*A gene probe. Nine out of twenty colonies reacted with the probe indicating their ability to produce the pediocin-like bacteriocin. These nine colonies were further tested for their antimicrobial spectrum, proteolytic inactivation and plasmid profile. It was found that a few of them were active against *Bacillus cereus, Micrococcus luteus* and *Listeria monocytogenes*. Their proteolytic inactivation showed that the antimicrobial compound was susceptible to proteinase K. Colony hybridization could thus enable rapid detection of pediocin and pediocin-like bacteriocin producers among a population of bacteriocinogenic strains.

Introduction

Lactic acid bacteria (LAB) are a fastidious group of aerotolerant, Gram-positive, non-sporing, non-motile, catalase-negative, carbohydrate-fermenting food-grade microorganisms that are generally regarded as safe (Geis *et al.* 1983; Nes *et al.* 2001). Besides other antimicrobial compounds, LAB produce bacteriocins that are gaining worldwide attention. Bacteriocins derived from food-grade microorganisms have a potential application as natural food preservatives. Bacteriocins are ribosomally synthesized, low molecular weight peptides that are heat stable and sensitive to proteolytic enzymes (Nes *et al.* 2001). Nisin, produced by *Lactococcus lactis*, is the only bacteriocin that is currently exploited for preservation of canned foods and dairy products (Delves-Broughton 1990). However, the use of nisin is limited due to its low solubility in water and narrow pH range (inactive at and above pH 7.0). Pediocin, a bacteriocin produced by different species of *Pediococcus* can be an ideal substitute for nisin in food preservation. Pediocin is an antilisterial, wide pH ranged, broad-spectrum, heat stable, class IIa bacteriocin (Ennahar *et al.* 2000; Rodriguez *et al.* 2002). Pediocin-producing pediococci are generally isolated from meat sources such as fermented sausages, fowl intestines, etc. (Marugg *et al.* 1992; Ennahar *et al.* 2000; Halami *et al.* 2000). However, isolation of pediocin producers from vegetable sources is limited (Bennik *et al.* 1997).

Conventionally, pediocin producers are isolated by microbiological assays that are time-consuming, tedious and ambiguous. Rapid methods such as colony immuno-blot, PCR, gene probe, etc. have been employed that help to overcome these inconveniences (Rodriguez *et al.* 1997; Martinez *et al.* 1998). In the recent past, production of intergeneric and interspecific pediocin PA-1/AcH by *Lactobacillus plantarum* and *P. parvulus* has been reported (Ennahar *et al.* 1996; Bennik *et al.* 1997). Likewise, pediocin-like antimicrobial peptides leucocin C and coagulin produced by *Leuconostoc mesenteroides* and *Bacillus coagulans* I_4, respectively, have been recorded (Le Marrec *et al.* 2000; Fimland *et al.* 2002; Rodriguez *et al.* 2002). These reports indicate that pediocin production is widely distributed among different genera and species of LAB and lactic acid-producing bacteria and hence there is a need to analyse a large number of bacteriocinogenic populations to isolate novel pediocin producers.

Table 1. Spectrum of antimicrobial activity of bacteriocin produced by *P. acidilactici* K7.

Organism	Strain	Antimicrobial activity (AU/ml)[A]
Bacillus cereus	F4433[a]	NI
Enterococcus faecalis	ATCC 344[b]	3200
Ent. faecium	MTCC 5153	1600
Lactobacillus amylovorous	NRRL B4552	NI
Lb. buchneri	NCIM 2357	1600
Lb. casei	CFR C67	NI
Lb. casei subsp. *casei*	CFR C40	NI
Lb. casei subsp. *casei*	CFR M10	1600
Lb. delbruecki subsp. *bulgaricus*	CFR 2028	NI
Lb. farciminis	CFR MD	3200
Lb. maltaromicus	MTCC 108	NI
Lb. plantarum	NCDO 955[c]	3200
Lb. plantarum	NCIM 2083	NI
Lb. viridescens	NCIM 2165	1600
Leuconostoc mesenteroides	NRRL B640	3200
Listeria monocytogenes	Scott-A[d]	12,800
L. monocytogenes	V7[e]	6400
Pediococcus sp.	CFR F7	NI
P. acidilactici	CFR F58	1600
P. pentosaceus	NRRL B11465	3200
Staphylococcus aureus	FRI 722[f]	1600

[A]Antimicrobial activity was determined by spot-on-lawn assay, NI: No inhibition.CFR: Department of Food Microbiology, CFTRI, Mysore; MTCC: Microbial Type Culture Collection, Chandigarh, India; NCIM: National collection of Industrial Microorganisms, Pune, India; NRRL: Northern Regional Research Laboratory, Peoria, IL, USA; a: strain obtained from Dr. J.M. Kramer, Central Public Health Laboratory, UK; b, d and e: strains obtained from Dr. A.K. Bhunia, Purdue University, USA; c: strain obtained from Dr. Bibek Ray, University of Wyoming, USA; f: strain obtained from Dr. E. Notermans, National Institute of Public health, Netherlands.

The present study deals with the isolation of pediocin PA-1-producing bacteria, characterization of the pediocin by molecular and microbiological techniques and development of colony hybridization as a rapid tool to enumerate viable pediocin producers in fermenting vegetables, in order to study the natural spread of pediocin producers.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used as indicators are shown in Tables 1 and 3. All LAB strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (HiMedia, Mumbai, India) at 37 °C. Pathogenic bacterial strains were propagated in nutrient broth (NB) and/or Brain Heart Infusion (BHI) broth (both obtained from Hi-Media) at 37 °C under shaking (200 rev/min).

Cucumber fermentation

Cucumbers (*Cucumis sativus*) were washed, cut into pieces and fermented in 6% NaCl at room temperature. Samples from this stock were drawn at different time intervals and tested for change in acidity (pH measurement), total lactic microflora (c.f.u./ml) on MRS agar plates by pour-plating and bacteriocinogenic strains detected by zones of inhibition on subsequent overlaying with indicator *Leuconostoc mesenteroides* NRRL B640.

Strain identification

A native strain of *P. acidilactici* was selected based on its strong antagonistic activity against the indicator B640. The strain was subjected to detailed identification and characterization by conducting various tests as described in *Bergey's Manual of Systematic Bacteriology* with certain modifications (Garvie 1986) and as described by Ramesh (2000). Various microbiological tests carried out were Gram staining, catalase test, CO_2 production from glucose measured in Durham tube, heat tolerance at 60 °C for 15 min and Scanning Electron Microscopy (Leo Electron Microscopy Ltd., UK). Biochemical tests such as carbohydrate fermentation, growth at different pH values, salt concentrations and temperatures, arginine and starch hydrolysis, gelatin liquefaction, etc. were also performed.

Characteristics of the antimicrobial compound

The test organisms were grown in MRS broth at 37 °C for 18 h and the culture filtrate (CF) obtained by centrifugation (10,000 rev/min for 10 min at 4 °C) was adjusted to pH 6.0 with 2 M NaOH and filtered through a 0.2 μm membrane filter (Millipore, India) and stored at –20 °C till further use. Antimicrobial activity of CF was tested against indicators shown in Table 3. Proteolytic inactivation of antimicrobial compounds present in CF was tested by using trypsin and proteinase K (SRL, Mumbai, India) and residual activity was assayed against the indicator strain B640.

Molecular techniques

For all molecular biology techniques, standard protocols were followed (Sambrook & Russell 2001). Native

Table 2. Summary of pediocin PA-1 purification.

Steps	Volume (ml)	Protein (mg/ml)	Activity (AU/ml)[a]	Total activity (AU)	Specific activity (AU/mg)	Yield (%)	Purification (fold)
Culture filtrate	1500	3.0	3200	4,800,000	1066	100	1
pH 2.0 extract	30	0.65	204,800	6,144,000	315,076	128	295

[a]Antimicrobial activity was tested by spot-on-lawn assay against the indicator B640.

Table 3. Antimicrobial spectrum, proteolytic inactivation and plasmid profile of bacteriocinogenic strains isolated by colony hybridization.

Properties	LAB isolates								
	b	e	i	k	l	m	o	q	s
Antimicrobial spectrum[a]									
B. cereus F4453	+	+	–	–	–	+	+	–	+
Leuc. mesenteroides B640	+	+	+	+	+	+	+	+	+
L. monocytogenes Scott-A	–	–	+	+	–	–	–	+	–
Micrococcus luteus NRRL B287	+	+	–	+	+	+	+	+	+
Staph aureus FRI 722	+	+	–	–	–	+	+	+	+
Proteolytic inactivation									
Trypsin	R	R	S	S	S	R	R	S	R
Proteinase K	S	S	S	S	S	S	S	S	S
Plasmid profile	Mp, 10 kb	Mp	Mp, 8 kb	Mp	Mp, 6 kb	Mp, 4 kb	Mp	Mp, 5 kb	Mp

[a]Antimicrobial spectrum was measured in terms of presence (+), absence (–) of zone of inhibition as carried out by agar well diffusion assay. R, Resistant; S, sensitive; Mp, high mol. wt. plasmid.

plasmids from the bacteriocin producers were isolated by the method of Anderson & McKay (1983) and analysed by agarose (0.8%) gel electrophoresis. The plasmid of CFR K7 was subsequently used for Southern hybridization. The PCR-generated *ped*A gene probe derived from the pediocin PA-1-producing strain of *P. parvulus* ATO 77 (Bennik *et al.* 1997) was DIG-labelled using the random primed DNA labelling kit (Boehringer Mannheim, Germany) and used as the *ped*A gene probe as reported previously (Ramesh *et al.* 2000).

For the amplification of the *ped*AB gene, PCR was carried out using the primers (Sigma–Aldrich, USA) 2A (5′ TAAGGATAATTTAAGAAGAAGGAG 3′) as forward primer and MR1 (5′ ATTTCTTAGAATT CTACAATATCCCC 3′) as reverse primer. The 2A and MRI primers are directed at positions 1043–1066 and 1651–1676 bp, respectively, of *Eco*RI/*Sal*I fragment derived from pSRQ11 (Marugg *et al.* 1992). The CFR K7 plasmid DNA was used as the template. The 16S rRNA gene was amplified by using the primers Kas.RNA.F (5′ CGACGTCGGCTCAGGATGAACGCTGGCGGC3′) and Kas.RNA.R (5′ GCTCTAGAGCGATTACTAG CGATTCCGACTTCG3′). The reaction was carried out in the GeneAmp PCR System 9700 (Perkin–Elmer, USA) thermocycler. The conditions for *ped*AB PCR were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 45 s, extension at 72 °C for 1 min and a final extension of 72 °C for 10 min. The rRNA gene was amplified using total cell lysate of K7 strain and the PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 45 s, extension at 72 °C for 1.5 min and a final extension of 72 °C for 15 min. *Taq* DNA polymerase (Bangalore Genei, India) was used at a concentration of 0.5 U for 50 μl reaction volume. The primers were used at a concentration of 1 μM each and dNTPs at a concentration of 0.2 mM each. The PCR amplicon was analysed by agarose gel electrophoresis. It was gel-eluted and subsequently purified using the gel elution kit (Qiagen, Germany).

The bacteriocinogenic colonies obtained during initial screening were transferred onto MRS plates and incubated at 37 °C for 12 h for the colonies to grow. The Hybond-nylon membrane (Amersham International, UK) was cut into the exact dimension of the plate and laid directly on the surface of the plate and then immediately removed. The colonies picked up on the membrane were subjected to lysis using the lysis buffer (Tris/HCl: 50 mM, glucose: 50 mM, lysozyme (Sigma, USA): 10 mg/ml) incubated at 37 °C for 45 min. The membrane was subsequently treated with 10% SDS followed by 0.5 M NaOH and 1.5 M NaCl, 0.5 M Tris/HCl (pH 7.0). The membrane was air dried, exposed to UV radiation and used for hybridization. The *ped*A gene-specific probe was used for colony hybridization. The conditions for hybridization were as described previously (Ramesh 2000).

Nucleotide sequencing

The gel-purified PCR product of the *ped*AB and 16S rRNA gene obtained from CFR K7 was ligated to the pTZ57R/T vector using 1.0 U of T_4 DNA Ligase (MBI, Fermentas, Lithuania) in a 20 μl reaction using the T-tail cloning kit (MBI). The reaction was carried out at 22 °C for 4 h and the ligation mixture was used for transforming chemically competent *E. coli* DH5α cells. Transformants were selected on X-gal, IPTG, ampicillin (50 μg/ml) agar plate and putative recombinants were selected by blue-white screening, followed by restriction analysis.

Unidirectional DNA sequencing was carried out by the dideoxy chain termination method using the M13F primer at the sequencing facility of Delhi University, South Campus; India. Clone Manager (Version: 4) was used for computational analysis of the nucleotide sequences. 16S rRNA gene sequences of K7 were analysed by using the program BLAST (Altschul *et al.* 1997).

Bacteriocin extraction and concentration

Bacteriocin produced by *P. acidilactici* CFR K7 was concentrated by cell-adsorption method as described by

Elegado *et al.* (1997) with certain modifications. For the production of bacteriocin, TGE (tryptone, glucose and yeast extract) broth was used. It consists of 1% each tryptone, yeast extract and glucose along with 0.005% each of $MgSO_4 \cdot 7H_2O$ and $MnSO_4$. The pH of the media was adjusted to 6.5 and after autoclaving, 0.2% of Tween 80 was added. The TGE medium (1.5 l) was inoculated with overnight-grown K7 strain and incubated at 37 °C for 20 h in static conditions. The fermented broth was heat inactivated at 70 °C for 30 min and the bacteriocin was adsorbed to the producer cells at pH 6.0. The bacteriocin-cell complex was precipitated by centrifugation (10,000 rev/min for 10 min at 4 °C) and the bacteriocin was desorbed at pH 2.0. The cells were separated by centrifugation at 15,000 rev/min at 4 °C for 30 min and the supernatant thus obtained was filtered through a 0.2 μm filter and dialysed in deionized H_2O using a 1000 MWCO dialysis bag (Spectra Por7, Fischer Scientific, USA). The dialysed material was freeze dried, dissolved in sterile double distilled water and the purity of the bacteriocin preparation was analysed by Tricine SDS-PAGE (Schägger & von Jagow 1987). The partially purified bacteriocin was used to study the effect of temperature on stability of antimicrobial compound at 40–100 °C for 30 min, 120 °C for 15 min and pH range of 2.0–9.0. Proteolytic inactivation of bacteriocin was studied by using the enzymes trypsin, chymotrypsin, protease, proteinase K and subtilin (SRL) at 1 mg/ml of bacteriocin preparation. The residual activity of bacteriocin was tested against the indicator strain B640.

Bacteriocin assay

The bacteriocinogenic strains were screened by overlaying the pour-plated LAB with the freshly grown ($\sim10^6$ c.f.u./ml) indicator strain *Leuc. mesenteroides* NRRL B640. The bacteriocin activity in the CF was tested by the agar well diffusion assay (Geis *et al.* 1983). The antimicrobial activity of pediocin PA-1 was determined by spot-on-lawn assay as described by Halami *et al.* (2000) and expressed in arbitrary units (AU/ml). Direct detection of antimicrobial activity of partially purified bacteriocin in SDS-PAGE was carried out by the method of Bhunia *et al.* (1987).

Nucleotide sequence deposition

Nucleotide sequences of partial *ped*AB and 16S rRNA genes are deposited in GenBank under the accession numbers: AY083244 and AY917122, respectively.

Results and discussion

Bacteriocinogenic LAB in cucumber

A large number of bacteriocinogenic colonies (% Bac$^+$) were enumerated over a time period to the maximum of

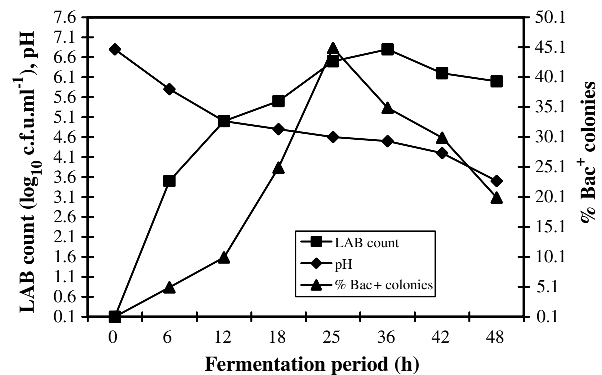

Figure 1. Growth of LAB, pH reduction and proportion of bacteriocinogenic strains active against *Leuc. mesenteroides* NRRL B640 in cucumber fermentation. (■) Viable cell count of LAB (log$_{10}$ c.f.u./ml); (♦) pH and (▲) bacteriocinogenic (Bac$^+$) colonies (%).

48 h of cucumber fermentation (Figure 1). It was observed that the total count of LAB increased from 1.778×10^6 c.f.u./ml after 6 h of fermentation to a maximum of 5.248×10^9 c.f.u./ml after 36 h of fermentation. This increase in bacterial count was concomitant with the drop in pH from 5.0 to 4.0, indicating active growth and production of acid by LAB. There was a steady increase in the population of LAB antagonistic to strain B640 as fermentation progressed. A maximum of 43% bacteriocinogenic colonies were recorded after 24 h of fermentation that declined subsequently to reach 21% after 48 h of fermentation. Such fermentations provide ample and diverse colonies to analyse for possible bacteriocin production.

Cucumber is a culinary vegetable that is often used in salads. Hence it is essential to analyse the microflora of cucumber, especially the bacteriocinogenic strains of LAB that can act as natural inhibitors of several foodborne pathogens. Cucumber fermentation by LAB under natural conditions has been shown, where glucose and fructose are used as the fermentable carbon sources (Daeschel & Fleming 1984).

Characteristics features of P. acidilactici *CFR K7*

A strain of *P. acidilactici* CFR K7 was selected based on diameters of the zones of inhibition being greater than 6 mm against the indicator strain B640. CFR K7 showed prominent zones of inhibition among more than 100 bacteriocinogenic LAB during initial screening. The CF of CFR K7 exhibited strong inhibition to the majority of the indicators tested and the antimicrobial activity varies from 1600 to 12,800 AU/ml (Table 1). The K7 exhibited antimicrobial activity against most of the species of genera *Leuconostoc*, *Lactobacillus* and *Pediococcus*. It also inhibited the growth of food spoilage LAB such as *Leuconostoc mesenteroides* and *Lb. viridescens* and foodborne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*. The K7 showed strong antilisterial activity against *L. monocytogenes* Scott-A, wherein

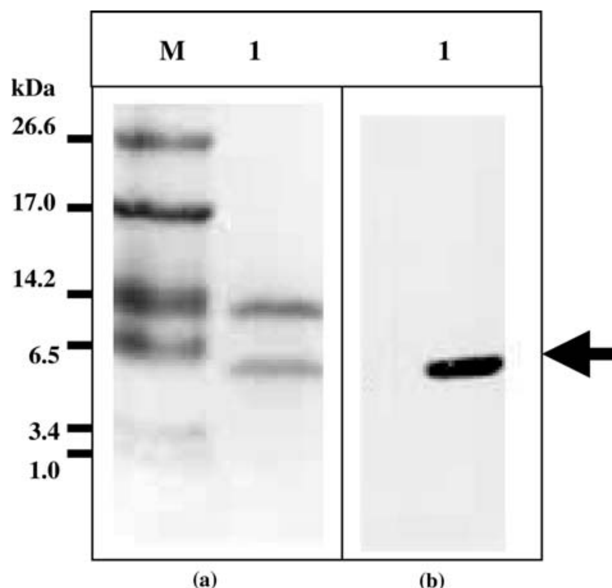

Figure 2. Tricine SDS-PAGE (18% acrylamide) separation and antimicrobial activity of partially purified pediocin PA−1. (a) Coomassie blue-stained tricine SDS-PAGE. Lane 1, pH 2.0 extract of pediocin PA-1 and M is a protein mol. wt. marker, ultra-low (Sigma, St. Louis, MO, USA). (b) Gel overlay assay of lane 1. Arrow indicates the zone of growth inhibition against B640. The inhibition of zone is corresponding to the 4.6 kDa size band.

an activity corresponding to 12,800 AU/ml was obtained (Table 1).

Since native isolate CFR K7 was potent bacteriocin producer, it was subjected to detailed characterization. Strain K7 was Gram-positive, tetracoccus and smooth (as revealed by SEM). It was homofermentative, catalase negative, showed production of ammonia from arginine, growth in the range of 37–50 °C, heat tolerance at 60 °C for 15 min, growth at an acidic pH of 4.4 and NaCl concentrations of 2.0–6.5. Strain K7 was able to ferment a wide range of sugars including arabinose, cellobiose, fructose, glucose, galactose, mannose, sucrose, trehalose and xylose. However, it was unable to ferment maltose, lactose, mannitol and melibiose. It was unable to liquefy gelatin and was able to hydrolyse starch. These results indeed coincide with those of Garvie (1986) enabling confirmation of this native isolate-K7 as *P. acidilactici*. Further BLAST results (nucleotide sequences spanning the region 767–1355 bp with respect to *E. coli* 16S rRNA) indicated maximum (99%) sequence homology with 16S rRNA gene of several strains of *P. acidilactici* (accession nos. AJ305322, AJ305320, M58833.1, AB018213, AB018214) followed by several other LAB including *P. pentosaceus* and *Lactobacillus* spp.

Biochemical and molecular features of the bacteriocin produced by CFR K7

The results on stepwise purification of the bacteriocin of CFR K7 are summarized in Table 2. It is evident that the partially purified bacteriocin showed almost 295-fold concentration and the purification yield was 128% over initial CF (Table 2). The Coomassie blue-stained acrylamide gel revealed two bands of ~4.6 and 12 kDa (Figure 2a). The smaller band of around 4.6 kDa exhibited antimicrobial activity against the indicator strain B640 (Figure 2b). This size band of bacteriocin is similar to that of pediocin AcM which was also found to be 4.6 kDa (Elegado *et al.* 1997). The high molecular weight (mol. wt.) band is likely to be a cell-surface-associated protein which is co-extracted with the bacteriocin at the acidic pH. The effect of temperature, pH and proteolytic enzymes was studied using the indicator strain B640. It was found that the heat-treated bacteriocin retained 100% activity when compared to the control. The bacteriocin was active in the pH range of 4–8. However, at an acidic pH of 2 and 3 and at an alkaline pH of 9.0, 50% activity was observed. After proteolytic treatment of the bacteriocin, there was a complete loss of antimicrobial activity. These results indicate that the bacteriocin is heat stable, acts at a wide pH range and is sensitive to proteolytic enzymes. The native plasmid isolated from the strain CFR K7 was subjected to Southern hybridization. It is evident that a 7.8 kb plasmid reacted with the DIG-labelled pediocin PA-1 (*ped*A) gene probe (Figure 3). However, the high mol. wt. plasmid and the chromosomal DNA failed to react with the gene probe (Figure 3b, lane 1). Thus, these results provide the evidence for the molecular basis for the presence of *ped*A gene on the 7.8 kb plasmid.

Based on the above biochemical properties and the strong antilisterial activity in combination with the *ped*A gene probe hybridization reaction, we conclude that the

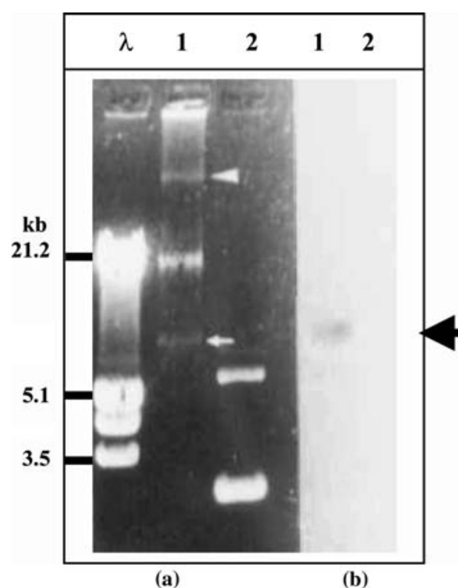

Figure 3. Detection of pediocin PA-1 encoding plasmid by Southern hybridization. (a) Agarose (0.8%) gel electrophoresis of plasmid DNA. Lane 1, plasmid of *P. acidilactici* CFR K7; lane 2, pBR322 plasmid; λ, lambda DNA *Eco*RI/*Hin*dIII marker. (b) Southern blot of lanes 1 and 2 probed with the *ped*A gene non-radioactive probe. Arrow indicates the probe hybridization with 7.8 kb plasmid.

strain of *P. acidilactici* K7 produces a bacteriocin named as pediocin PA-1. The deduced amino acid sequence encoded by the *ped*A gene of K7 indicates the presence of two disulfide bridges that probably contribute to heat stability and pH tolerance. Similar properties were observed in case of well characterized pediocin AcH/PA-1 (Rodriguez *et al.* 2002).

Previously, pediocin-producing strains of bacteria have been isolated from various sources. In the present study, we used fermented cucumbers as a source for the isolation of pediocin PA-1-producing strain of *P. acidilactici*. Pediococci are often associated with meat fermentation, however in plant material they are found as saprophytes (Garvie 1986). In many of the studies, pediocin has been purified from complex medium such as MRS. However, in the present study we have used TGE broth, a food-grade medium for the production of bacteriocin. By using cell adsorption–desorption technique (Elegado *et al.* 1997) it was possible to partially purify the pediocin of strain K7 in a quantifiable amount in two steps and thus reducing the number of steps involved in bacteriocin purification.

Bacteriocin produced by P. acidilactici *is pediocin PA-1*

Nucleotide sequence analysis of the 540 bp fragment of *ped*AB gene indicated 100% homology with pediocin AcH/PA-1 and its partial *ped*B counterpart. It further substantiated the fact that at position 201 of *ped*B gene sequence, CFR K7 is similar to *ped*B of *P. acidilactici* and not *P. parvulus*/*Lb. plantarum* type (Mora *et al.* 2000). This clearly indicates that the native isolate of *P. acidilactici* has a pediocin AcH/PA-1-like bacteriocin production operon.

The results of nucleotide sequence data of *ped*AB gene of strain CFR K7 in combination with the nucleotide sequence data of pediocin operon available in GenBank suggest that the genes involved in the production of pediocin are highly conserved. However, there is a single nucleotide difference among *ped*B and *ped*D genes in the intergeneric/interspecies pediocin-producing strains (Mora *et al.* 2000). Such unique nucleotide exchanges can be used to discriminate the intergeneric and interspecies pediocin AcH/PA-1 producers of different origin.

Rapid detection of pediocin producers by colony hybridization

Twenty representative LAB colonies isolated from cucumber fermentation that exhibited antimicrobial activity on the lawn of strain B640 were aseptically transferred onto a MRS plate and labelled 'a'–'t'. Upon probing with the *ped*A gene probe, nine (45%) showed a strong reaction, indicating that these bacteria are pediocin producers (Figure 4). Colonies b, i, k, o, q, and s reacted with higher intensity, indicating that these colonies possess the genetic determinant coding for pediocin AcH/PA-1 production. Colonies e, l and m showed a faint reaction. These colonies probably produce a bacteriocin that is similar to the pediocin AcH/PA-1. Although all the 20 colonies had exhibited antimicrobial activity against strain B640, only nine colonies reacted with the *ped*A gene probe indicating the specificity of the probe.

The CF of the putative pediocin-like bacteriocin producers isolated by colony hybridization showed zones of inhibition against several pathogens (Table 3). Almost all the strains inhibited the growth of *M. luteus*, while only three of the strains inhibited the growth of Scott-A. Majority of the strains inhibited *Staph. aureus* and *Bacillus cereus*. The CF of all the strains, on treatment with proteinase K showed no zones of inhibition against strain B640, indicating the proteolytic nature of the antimicrobial compound. Strains b, e, m, o and s showed zones of inhibition even after treatment with trypsin, which coincides with the observation made in case of pediocin PD–1 (Green *et al.* 1997). Pediocin PD-1 is a bacteriocin produced by *P. damnosus* PD-1, which is known to be resistant to pepsin, papain, trypsin and α-chymotrypsin and sensitive to proteinase K. Pediocin PD-1 was found to be 2866.87 + 0.4 Da in size, with p*I* of ca. 9.0. Alignment of the amino acid sequence revealed areas of homology to lantibiotics such as plantaricin C, actagardine and mersacidin (Bauer *et al.* 2004). All the native strains harbour different sizes of plasmids ranging from 4 kb to high mol. wt. indicating that these strains differ from each other.

Since pediococci are known to produce pediocin, we used the Southern hybridization technique to detect the

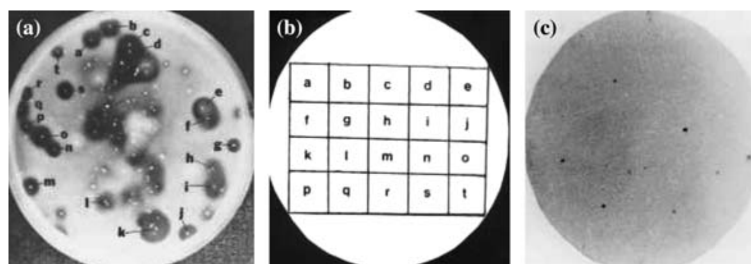

Figure 4. Rapid detection of pediocin PA-1-like bacteriocin producer from fermenting cucumber by colony hybridization. (a) Bacteriocinogenic colonies of LAB (24 h of fermentation) exhibiting zones of inhibition against B640. Bac^{+} colonies spotted on MRS agar plate. (b) Position of Bac^{+} LAB colonies spotted on MRS agar plate. (c) Colony hybridization using DIG-labelled pediocin PA-1 (*ped*A) gene probe.

presence of pediocin genes. In our experiment, the 7.8 kb plasmid reacted with the *ped*A gene probe indicating the presence of genes involved in pediocin production. Previously it has been shown that pediocin production is a plasmid-linked phenotype and the plasmid size varies from strain to strain (Marugg *et al.* 1992; Halami *et al.* 2000). In our studies, the use of the Southern hybridization technique facilitated the easy detection of the 7.8 kb bacteriocin-encoding plasmid, eliminating the high mol. wt. plasmid and chromosomal DNA that are not involved in bacteriocin production. Therefore, DNA probe-based detection complemented the conventional curing experiment. Previously, Bhunia *et al.* (1994) had demonstrated the use of the Southern hybridization technique for the identification of bacteriocin-encoding plasmids. DNA probes have been used earlier to detect silage colonization by *Lactobacillus* and *Pediococcus* strains and bacteriocinogenic LAB (Rodriguez *et al.* 1997; Martinez *et al.* 1998). In these studies, the specificity of the DNA probe was tested using pure cultures, but in the present investigation, the pediocin PA-1 probe has been used directly to screen a heterogeneous population of antagonistic cultures isolated from naturally fermented cucumber.

Pediocin belongs to the class IIa family of bacteriocins that are characterized by the presence of YGNGV and CXXXXCXV sequence motifs at their N-terminal halves (Nes *et al.* 2001; Rodriguez *et al.* 2002). Such conserved consensus sequences enable designing of primers and probes for the isolation of class IIa bacteriocins from fermenting populations. In the present investigation, PCR-generated *ped*A gene probe was specifically designed by choosing the 1043–1301 bp region of the pediocin operon (Marugg *et al.* 1992), which corresponds to the complete pre-peptide coding region of the pediocin gene. Among class IIa bacteriocins, there is a high degree of homology ranging from 34 to 80% sequence identity (Rodriguez *et al.* 2002). These characteristic features at molecular level are perhaps also involved in hybridization of the gene probe with similar bacteriocin-encoding genes. Hence, preliminary screening of bacteriocinogenic strains using a DNA probe and subsequently characterizing them can expedite the process of isolation of potent bacteriocinogenic strains in natural populations. The use of DNA probes in colony hybridization specifically serves as a versatile tool to detect viable bacteriocinogenic strains, overcoming the drawback of PCR and *in situ* hybridization that detect non-viable cultures too.

Since colony hybridization does not require previous isolation of DNA like other DNA-based techniques, it allows direct analysis of bacteria from a mixed culture. Limited research has been directed towards the use of such techniques and this has so far been demonstrated only in pure cultures. In this study, we provide evidence for rapid enumeration of pediocin-like bacteriocin producers in naturally fermented cucumber. Such techniques serve as rapid tools for direct screening of bacteriocinogenic strains, while searching for novel bacteriocins.

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