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Detection and Characterization of Pediocin PA-1/AcH like Bacteriocin Producing Lactic Acid Bacteria

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Abstract Fifty-five bacteriocinogenic lactic acid bacteria (LAB) isolated from seven different sources. Eight isolates were found to produce pediocin PA-1 like bacteriocin as detected by *ped*B gene PCR and dot-blot hybridization. The culture filtrate (CF) activity of these isolates exhibited strong antilisterial, antibacterial activity against tested food-borne pathogens and LAB. The identification and genetic diversity among the selected LAB was performed by conventional morphological and molecular tools like RFLP, RAPD, and 16S rDNA gene sequencing. The isolates were identified as, 1 each of *Pediococcus acidilactici* Cb1, *Lactobacillus plantarum* Acr2, and *Streptococcus equinus* AC1, 2 were of *P. pentosaceus* Cb4 and R38, and other 3 were *Enterococcus faecium* Acr4, BL1, V3. Partial characterization of the bacteriocins revealed that the peptide was heat-stable, active at acidic to alkaline pH, inactivated by proteolytic enzymes, and had molecular weight around 4.6 kDa and shared the properties of class IIa pediocin-family. The bacteriocin production at different temperatures, pH, and salt concentrations was studied to investigate the optimal condition for application of these isolates as a starter culture or as a biopreservative in either acidic or non-acidic foods.

Introduction

The pediocin PA-1/AcH (pediocin PA-1) represent a class IIa bacteriocin of low molecular weight, unmodified antilisterial peptides with a consensus motif of YGNGVXC at their N-terminal end [12]. Among all the class IIa bacteriocins, pediocin PA-1 is widely distributed and is more potent in inhibiting the growth of several pathogens associated with food spoilage and food related health hazards and hence can be a potential food bio-preservative agent [14].

Pediocin PA-1 is a plasmid encoded bacteriocin initially characterized from the strains of *P. acidilactici* PAC 1.0 [8]. Subsequently, other species viz *P. pentosaceus*, *P. parvulus* and other genera viz. *Lactobacillus plantarum* and *B. coagulans* were reported for the production of same bacteriocin where in different environmental conditions are known to influence bacteriocin production [3, 5, 7, 10]. The gene organization and sequences of pediocin PA-1 operon were found to be highly conserved and resides on plasmid size that ranges from 9 to 14 kb [10]. These reports are in concurrent observation that distribution of pediocin PA-1 operon among different bacteria took place by integration into the native plasmids [7]. In order to study such transfer, there is a need for detection and characterization of large number of pediocin PA-1 producers from different sources.

Although pediocin PA-1 producers are reported from different LAB, specific isolation of intergeneric and interspecific pediocin PA-1 like bacteriocin producers are not reported. Hence, in this study an attempt was made for the rapid detection of pediocin PA-1 like bacteriocin producers in different genera and species of LAB by using molecular tools. Influence of cultural conditions for the production of pediocin PA-1 like bacteriocin was also investigated.

Materials and Methods

Bacterial Strains and Maintenance

Standard pediocin PA-1 producers viz. *Pediococcus acidilactici* PAC1.0 [8], *P. acidilactici* K7 [6] and enterocin A

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producer *Enterococcus faecium* MTCC 5153 (MTCC, Chandigarh, India) were used in this study. All the above LAB cultures as well as *P. acidilactici* ΔK7, *P. acidilactici* ΔPAC1.0 (plasmid cured strains, obtained by novobiocin treatment), *Leuconostoc mesenteroides* NRRL B640 (NRRL, Peoria, USA) and the LAB isolates of the study were grown in de Man, Rogosa and Sharpe (MRS) broth or on MRS agar (Hi Media, Mumbai, India) at 37°C in static condition. The food-borne pathogenic indicator strains viz. *Listeria monocytogenes* ScottA, *L. innocua* FB 21, and *L. murrayi* FB 69 (obtained from Dr. AK Bhunia, Purdue University, USA); *Aeromonas hydrophila* NRRL B445; *Yersinia entericolitica* MTCC859 and *Escherichia coli* MTCC118, *Staphylococcus aureus* FR1722, *Salmonella typhi* FB231, and *S. paratyphi* FB254 (from Dr. E. Noterman, National Institute of Public health, Netherlands), were grown in Nutrient broth or BHI broth (Hi Media, Mumbai) at 37°C under shaking (200 rpm). The above mentioned strains were maintained at −40°C in lactobacilli MRS media and BHI or Nutrient media with 40% glycerol (v/v). Before being used, strains were propagated twice in their respective broth.

Isolation of Bacteriocinogenic LAB

The isolation of antilisterial bacteriocin producing LAB from fermented vegetable sources like carrot, cucumber, beans, and betel leaves was performed using ScottA as indicator described previously [6]. The other sources like fermented milk (curd) and chicken intestine sample were diluted and pour plated and observed for zone of inhibition against ScottA, and further characterized as described previously [6].

PCR Amplification of Pediocin PA-1 Genes

Total DNA from LAB was isolated as described by Mora et al. [11]. All the oligonucleotide primers were obtained from Sigma-Aldrich (Bangalore, India) and the PCR components were from Bangalore GeNei (Bangalore). The *ped*B gene was amplified by using primers, *ped*BF (5′GG TGATTTTATGAATAAGACTAAGTCG3′) and *ped*BR (5′CCCCTTTATCAGTACTATTGGCTAGGC3′) positioned at 3488–3514 and 3823–3849 as per the sequence of pSMB74 of *P. acidilactici* H (Accession number-U02482). The standard procedure for PCR amplification was followed as described by Sambrook and Russell [15] with annealing at 60°C. Similarly, *ped*AB gene was amplified as described earlier [6]. All the PCR amplicons were analyzed by 1.5% agarose (SRL, Mumbai, India) gel electrophoresis.

DNA Dot-Blot Hybridization

The PCR product of *ped*B gene obtained from *P. acidilactici* PAC1.0 was labelled with digoxigenin-dUTP using random primed DNA labeling kit (Roche Chemicals, Germany) and used as a probe for dot-blot analysis. Ten microlitre of total DNA (approximately 25–50 ng μl^{-1}) of test culture was heat denatured, spotted on a Hybond Nylon membrane (Amersham International, UK) according to the method described earlier [15] and hybridized using probe. Hybridization and stringency washes were carried out at 42°C according to manufacturer instructions (Roche chemicals, Germany).

Phenotypic and Biochemical tests

The Gram-staining, catalase, fermentation of carbohydrate viz. 1% each of glucose, lactose, maltose, sucrose, mannitol, sorbitol, etc., gas production from glucose was performed as per standard microbiological methods. Growth of test cultures in MRS broth at different temperatures (10, 37, and 45°C) was evaluated upon incubation for 16 h. Similarly, growth in MRS broth containing 5 and 8% NaCl and at pH (4, 8, and 10) was also tested.

RAPD, RFLP, and 16S rDNA Gene Sequencing

Random Amplified Polymorphic DNA (RAPD) PCR of total DNA was carried out by primer M13 (5′GAG-GGTGGCGGTTCT3′) in a 25 µl reaction volume as described earlier [17]. Digestion of 16S rDNA gene PCR product with *Hae*III and *Alu*I enzymes (Bangalore GeNei, Bangalore) for Restriction fragment length polymorphism (RFLP) analysis was performed. The primers and the PCR conditions used for amplification were followed as described earlier [13]. DNA sequences of 16S rDNA PCR product was sequenced at the sequencing facility of Vimta Labs (Hyderabad, India). The gene sequences obtained were analyzed using the BLAST search programme [1].

Antibacterial Activity Assay

The test cultures were grown in MRS broth at 37°C for 16 h under static condition. The cultures were centrifuged at 9000 rpm in 4°C for 15 min and the culture filtrate (CF) was collected, filtered through 0.4 μ filter (Millipore) and stored at 4°C until further use. The inhibitory effect of the CF was tested against food-borne pathogens and LAB cultures by spot-on-lawn assay [4].

Characteristics of Antimicrobial Compound

The CF of the test culture was subjected to treatment with different proteolytic enzymes such as proteinase K, papain, trypsin, pepsin (SRL) at a final concentration of 1 mg ml^{-1}, reducing agents (conc. 10%) like β-mercaptoethanol (SRL) and Dithiothreitol (DTT) (SRL) were also

used. Reaction mixture was incubated at 37°C for 2 h and residual activity was determined using ScottA, as described previously [13]. Similarly, stability of CF at different temperature and varying pH was also tested as above. The chloroform extracted bacteriocin from CF was subjected for bioassay by Tricine–SDS-PAGE [16] and overlaid with ScottA.

Bacteriocin Production at Different Temperatures, pH, and NaCl Concentration

MRS broth with pH of 4, 8, and 10 (adjusted by HCl or NaOH), as well as MRS with 4 and 8% sodium chloride (w/v) (SRL) was prepared and inoculated with 1% freshly grown test cultures and allowed growth for 16 h at static conditions at 37°C. Growth (OD 600 nm) and bacteriocin production of test cultures in MRS broth at different temperatures (15, 37, and 50°C) was also studied as described above. Antilisterial activity expressed as arbitrary unit per ml (AU ml^{-1}) and defined as the highest dilution of test sample exhibiting the zone of inhibition against indicator ScottA.

Results and Discussion

Detection of Putative Pediocin PA-1 like Bacteriocin Producing LAB

In order to study intergeneric and interspecific pediocin PA-1 producers, we have screened large number of antilisterial bacteriocin producing LAB. Among the screened sources, the LAB isolated from vegetables displayed strong antilisterial activity. From each representative source, the cultures with high activity were selected and subsequently tested for the presence of immunity protein of pediocin PA-1 (*ped*B) gene by PCR. Among 55, 8 cultures gave expected amplicons of 362 bp for *ped*B and 600 bp for *ped*AB genes. The results of CF activity against ScottA and *ped*B PCR analysis of the selected native isolates is shown in Fig. 1a, b. PCR results were additionally confirmed by dot-blot hybridization using *ped*B gene probe. As expected, cultures K7, BL1, Acr2, Acr4, Cb1, Cb4, V3, AC1, and R38 gave positive signal suggesting a conserved pediocin PA-1 gene in native isolates, whereas, *E. faecium* MTCC 5153 and ΔPAC1.0 did not react with the probe (data not shown). The detection of pediocin PA-1 by molecular tools was earlier reported in *P. parvulus* [3] and *P. acidilactici* [9].

Characteristics of Native LAB

All the selected isolates were Gram-positive, catalase negative and cocci in shape except the isolate Acr2, which was rod shaped. The gas production was observed only for Acr2 and V3 isolate. The isolate Acr2, was unable to grow at 45°C, 8% NaCl and at pH 10. Similarly AC1 isolate was unable to grow in 8% NaCl concentration, 10 and 45°C temperatures and also at pH 4 and 10. The isolates were able to ferment different carbohydrates tested, except AC1 which could not utilize lactose. The other isolates were able to grow at all the parameters used. These results suggested that, the isolates had distinct characteristic features.

Molecular Typing of Putative Pediocin PA-1 Producers

In order to differentiate the isolates among each other and also from native *P. acidilactici* K7, RAPD PCR was performed. RAPD showed five different banding profiles indicating, the selected isolates were different from each other. Similarly, RFLP also showed variability at their 16S rDNA gene (Fig. 2a, b). For species level identification, 16S rDNA gene sequencing followed by BLAST analysis was performed. DNA sequence homology in combination with the results of physiological and biochemical tests, the putative pediocin producers were identified as follows—*Streptococcus equinus* AC1, *Pediococcus acidilactici* Cb1, *Pediococcus pentosaceus* Cb4 and R38, *Lactobacillus plantarum* Acr2, *Enterococcus faecium* Acr4, BL1, and V3. The 16S rDNA gene sequences (~700 bp) have been deposited in the GenBank database under the Accession numbers GU222444–GU222450 for the LAB strains AC1, Cb1, Cb4, Acr2, V3, BL1, and Acr4, respectively. The bacteriocin producing LAB isolates reported in this study are deposited in the National Collection of Industrial Microorganisms (NCIM) at the National Chemical Laboratory, Pune, India.

Antibacterial Spectrum and Properties of Bacteriocin

All test isolates were studied for their ability to inhibit various food-borne pathogens as well as closely related LAB species like *E. faecium* 5153 and *Leuconostoc mesenteroides* NRRL B640. The tested isolates were able to inhibit all the *Listeria* spp., mutants of K7 and PAC 1.0 as well as Gram-negative *Aeromonas* and *Yersinia* sp, with an inhibition zone size of around 10–18 mm. Isolates *St. equinus* AC1, *Lb. plantarum* Acr2, *E. faecium* Acr4, and *P. pentosaceus* R38 were also able to inhibit Gram-positive *Staphylococcus aureus.* None of the isolates inhibited *P. acidilactici* K7, PAC 1.0, as well as *Escherichia coli* and *Salmonella typhi*. Inhibitory spectra of pediocin PA-1 to selected Gram-positive bacteria were earlier reported [12, 14]. The Gram-negative *Aeromonas hydrophila* B445 was similarly inhibited by pediocin SA1 [2]. In general, an antibacterial spectrum of Cb4, Acr2, Acr4, BL1, and V3

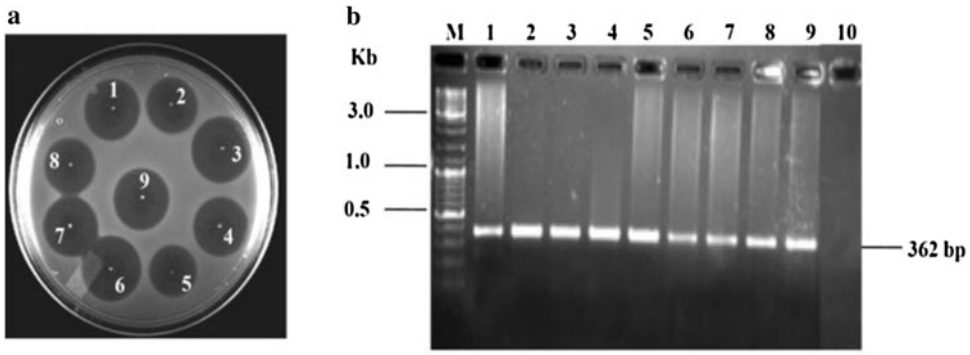

Fig. 1 Detection of antilisterial activity of native LAB isolates by spot-on-lawn assay (**a**), *ped*B gene specific PCR (**b**). **a**, **b** *1* K7, *2* Cb1, *3* Cb4, *4* R38, *5* AC1, *6* Acr4, *7* Acr4, *8* BL1, *9* V3. **b** *10* MTCC 5153 (Negative control), *M* 10 Kb molecular marker

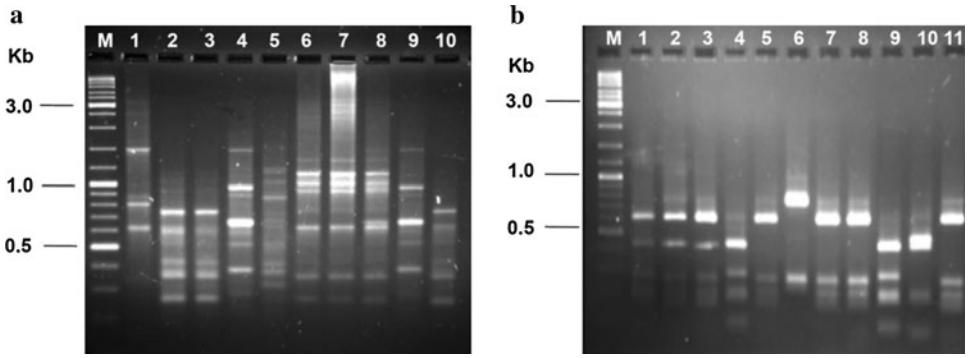

Fig. 2 Differentiation of the native pediocin PA-1 like bacteriocin producers by RAPD PCR (**a**) and RFLP of 16S rDNA gene digested with *Hae*III and *Alu*I (**b**). **a** *Lane 1* AC1, *2* K7, *3* Cb1, *4* Cb4, *5* Acr2, *6* BL1, *7* V3, *8* Acr4, *9* R38, *10* PAC1.0 **b** *Lane 1* K7, *2* PAC1.0, *3* Cb1, *4* Cb4, *5* BL1, *6* Acr2, *7* Acr4, *8* V3, *9* R38, *10* AC1, *11* MTCC 5153. *M* is a 10 Kb molecular marker (GeNei, Bangalore) in both the gels

was found to be different than the pediocin PA-1 producing in *P. acidilactici* K7.

The protease sensitivity and inactivation by reducing agents suggested the proteinaceous nature and involvement of disulfide bridge, respectively, of the AMC. Optimum activity of CF for most of the isolate was found to be at pH 7–8 and temperature between 40 and 80°C. However, at pH 2–4 and temperature at 100 and 121°C, the activity was reduced to ~50%. Antilisterial activity was retained at even pH 10 and at 90°C suggesting heat stable and wide pH range AMC. Upon Tricine SDS-PAGE analysis, all isolates had the active peptide of 4.6 kDa (data not shown). The above reported observations for the selected isolates are similar to that described for pediocin PA-1 [14].

Effect of Cultural Conditions on Production of Bacteriocin

The bacteriocin production for all the cultures was more at 37°C when compared to 15 and 50°C. All the strains of *E. faecium* V3, Acr4, BL1 were able to grow and produce bacteriocin at all the temperatures, pH, and NaCl concentration, this could be due to the fact that *E. faecium* has wider adaptability to environment. The isolate *P. acidilactici* K7, Cb1, and *Lb. plantarum* Acr2 were able to grow at 15 and 37°C. The isolate *St. equinus* AC1 was unable to grow at 15 and 50°C, pH 4 and 10, and at 8% NaCl concentration. Similarly, the isolate *Lb. plantarum* Acr2 was unable to grow at pH 4 and 10 and did not produce bacteriocin (Fig. 3). The bacteriocin production is greatly influenced by the nutrients, temperature, pH, NaCl concentration [4]. The optimum condition for pediocin AcH, SA-1, and other class IIa bacteriocins was found to be at temperature 30–35°C, pH 5–7, and NaCl 1.5–3% [12]. Results obtained suggested that these native isolates could be used as a protective culture in acidic foods like pickles and yogurt, as they exhibited good growth and bacteriocin production at different cultural conditions.

In conclusion, we detected the presence of pediocin PA-1 gene cluster and PA-1 like bacteriocin properties in *E. faecium* and *St. equinus* besides *P. pentosaceus* and *Lb. plantarum* of vegetable and dairy origin. The molecular typing tools have proven to be useful in differentiation, characterization, and identification of LAB in spite of their high heterogeneity and phylogenetic inter-mixing. These cultures can be further used to study the pediocin PA-1 operon integration, revealing the possible mechanism of horizontal operon transfer as reported for *B. coagulans* I_4 and *Lb. plantarum* 423 [7, 18]. We are presently investigating the flanking regions of the operon to discover the

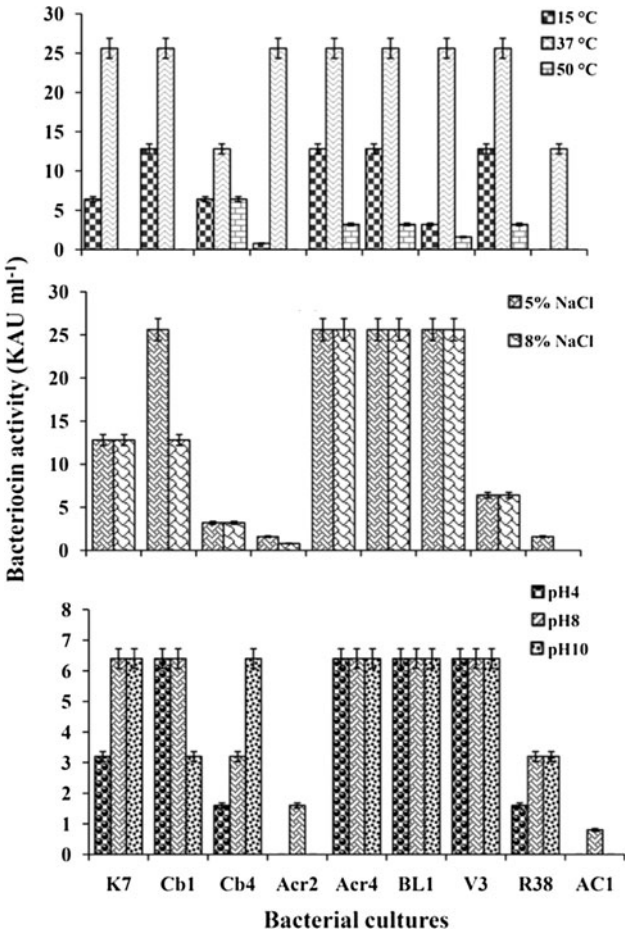

Fig. 3 Production of bacteriocin by native isolates at various temperatures (**a**), NaCl concentration (**b**), and pH (**c**). Bacteriocin production indicated in *bars* and was determined by CFS activity (KAU ml^{-1}) against ScottA. Results are the mean of ±SD ($n = 2$ trails)

novel mobile genetic elements involved in such recombination events. Hence, Pediocin PA-1 produced by LAB other than *P. acidilactici* can be of industrial significance in different food systems because of their wider environmental adaptability.

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