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A 'bacteriocin PCR array' for identification of bacteriocin-related structural genes in lactic acid bacteria ☆

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ABSTRACT

Bacteriocins have been identified in many strains of lactic acid bacteria (LAB) which are a source of natural food preservatives and microbial inhibitors. Our objectives were to use a PCR array of primers to identify bacteriocin structural genes in Bac$^+$ LAB. DNA sequence homology at the 5′- and 3′-ends of the various structural genes indicated that non-specific priming may allow PCR amplification of heterologous bacteriocin genes. Successful amplification was obtained by real-time PCR and confirmed by melting curve and agarose gel analysis. Sequence information specific to targeted bacteriocin structural genes from the intra-primer regions of amplimers was compared to sequences residing in GenBank. The bacteriocin PCR array allowed the successful amplification of bacteriocin structural genes from strains of *Lactobacillus*, *Lactococcus*, and *Pediococcus* including one whose amino acid sequence was unable to be determined by Edman degradation analysis. DNA sequence analysis identified as many as 3 bacteriocin structural genes within a given strain, identifying ten unique bacteriocin sequences that were previously uncharacterized (partial homology) and one that was 100% identical to sequences in GenBank. This study provides a rapid approach to sequence and identify bacteriocin structural genes among Bac$^+$ LAB using a microplate bacteriocin PCR array.

1. Introduction

Bacteriocins are ribosomally-synthesized pore-forming antimicrobial peptides that are inhibitory to sensitive organisms (Venema et al., 1995). They are produced by many genera of bacteria, including many strains of lactic acid bacteria (LAB). Since the LAB are generally-recognized-as-safe (GRAS) for use in foods, the bacteriocins produced by them have been proposed for use in the preservation of food products by eliminating or controlling spoilage microorganisms and pathogens (De Vuyst and Vandamme, 1994). In the past 25 years, hundreds of papers have been published describing and characterizing bacteriocin-related inhibitory activities from LAB isolated from foods and traditional fermentations. Many bacteriocins of LAB have now been identified and characterized, both biochemically and genetically (Cleveland et al., 2001).

Companies that provide freeze-dried or frozen cultures to processors are also actively involved in isolating LAB and depositing them in their own collections, which in turn are distributed in fermented or cultured food products. The widespread natural distribution, commercial re-distribution, and research interest in Bac$^+$ LAB presents a challenge to researchers planning analyses from newly isolated Bac$^+$ strains as these may be redundant isolates already studied. Hequet et al. (2007) examined various meat isolates and found that *Lactobacillus curvatus* 2711 and *Leuconostoc pseudomesenteroides* 2733 were able to produce bacteriocin in a liquid medium that simulates meat growth conditions. After inoculated challenge studies showing inhibition of *L. innocua*, they examined the bacteriocin by HPLC purification, mass spectrometry, and partial amino acid sequence analysis only to find that the broad spectrum bacteriocin produced by *Lb. curvatus* 2711 was actually sakacin X, confirmed by PCR analysis of the various sakacin genes (*sak*X, *sak*T$_\alpha$, *sak*T$_\beta$) using sequence information already available in the literature (Vaughan et al., 2003).

The most common approach to determine the identity of new found bacteriocins has been random testing by PCR with primers targeting one or more suspect bacteriocins (Akcelik et al., 2006; Reminger et al., 1996; Rodriguez et al., 1995). Knoll et al. (2008) and Omar et al. (2008) both employed the use of multiple bacteriocin-related gene targets in characterizing lactic acid bacteria isolated from the malolactic fermentation of wines or from fermented maize, respectively. Both groups used PCR primers targeting many of the 20 or more bacteriocin-related genes clustered in 5 different operons in *Lactobacillus plantarum*, a common isolate in the two types of fermentations they examined.

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The various genes encode bacteriocin and immunity proteins, ABC transport systems, signal-transducing systems, and others of unknown function involved with bacteriocin synthesis.

The use of PCR techniques can readily identify bacteriocin genes in bacteriocinogenic LAB. Yi et al. (2010) used a variation of 'colony PCR' to facilitate the screening of colonies for class IIa bacteriocin-producing LAB. They used degenerate primers based on the conserved N-terminal regions found in many class IIa bacteriocins and specific downstream primers to design PCR reactions resulting in large amplimers specific for pediocin, enterocin, and plantaricin (Yi et al., 2010). Wieckowicz et al. (2011) used a PCR assay with a custom panel of bacteriocin-related primers to screen metagenomic DNA preparations obtained from the microflora of Polish artisanal cheeses and found they were able to identify class IIa bacteriocin sequences in LAB as well as from non-LAB sources.

Similarly, we examined the use of a "bacteriocin PCR array" to assist in identifying if Bac^+ isolates are novel or redundant (Macwana and Muriana, 2006). In this study, the DNA from Bac^+ strains and food isolates were subjected to a bacteriocin-specific PCR array in individual reactions with primers representing forty-two known structural genes of bacteriocins from LAB. Sequencing of the amplimers followed by sequence analysis helped to determine if the sequences had identity with others currently in GenBank or were unique sequences.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are described in Table 1. Bacteriocin-producing (Bac^+) lactic acid bacteria (LAB) were isolated previously from food samples obtained from local supermarkets (Garver and Muriana, 1993; Macwana and Muriana, 2012; Table 1). LAB were grown in MRS broth (Difco Laboratories, Detroit, Mich.) or on MRS agar and incubated at 30 °C under static conditions. Cultures were transferred (0.1% inoculum) 2–3 times in MRS broth and incubated at 30 °C for 24 h before use. LAB cultures were maintained as frozen stocks at −80 °C in MRS broth with 10% glycerin.

2.2. Identification of bacteriocin-producing LAB

The identification and biochemical characterization of Bac^+ isolates was confirmed using API 50 CH panels (bioMerieux Inc., Durham, NC). The manufacturer's directions were modified by using overnight growth in broth instead of cells recovered from agar plates. The broth cultures were harvested by centrifugation and the supernatant fraction was discarded. The cell pellet was washed twice with 10 ml of Carbohydrate Lactic Acid (CHL) broth and the final resuspension was aliquoted into the API 50 CH panels. The panel strips (consisting of various substrates) were incubated anaerobically (GasPak) at 30 °C for 48 h. If a carbohydrate was fermented, the indicator turned from purple to yellow, indicating a positive reaction; negative reactions did not show a color change and remained purple. The results were then scored against the manufacturer's computerized database to obtain results.

Table 1
Bacterial strains used in this study.

Bacterial strains	Source	Reference
Lactobacillus delbrueckii subsp, *lactis* 4797	FAPC culture collection	Garver and Muriana (1993)
Lactobacillus curvatus FS47	Ground beef	Garver and Muriana (1993)
Pediococcus acidilactici PAC1.0	FAPC culture collection	Marugg et al. (1992)
Lactococcus lactis FS56	Mushrooms	Garver and Muriana (1993)
Lactococcus lactis FS97	Vegetables	Garver and Muriana (1993)
Lactococcus lactis FS92	Raw pork	Garver and Muriana (1993)
Pediococcus acidilactici Bac3	Ground turkey	Macwana and Muriana (2011)
Lactobacillus sakei JD1	Ground pork	Macwana and Muriana (2011)
Lactococcus lactis RP1	Raw pork	Macwana and Muriana (2011)

2.3. PCR array for bacteriocin structural genes

The coding strand sequences for forty-two known LAB bacteriocin structural genes were retrieved from GenBank (Table 2). Primers were designed using the Primer Express Software (Applied Biosystems, Foster City, CA). The criteria for the primer design included optimum length of 25 bp and melting temperature (T_m) of 58 °C–60 °C. A total of 42 pairs of primers were designed from the coding strands for as many bacteriocin structural genes as possible targeting *Lactobacillus* spp., *Pediococcus* spp., *Lactococcus* spp., and *Leuconostoc* spp. Prior to PCR, the DNA from the Bac^+ strains isolated by our bacteriocin screening process was extracted by the BAX^{Tm} procedure (Qualicon, Wilmington, DE): 5 µl of overnight culture was mixed with 200 µl of BAX^{Tm} lysis reagent containing protease, and cell lysis was performed by incubating this mixture at 55 °C for 60 min and then 95 °C for 10 min. The PCR reaction mix consisted of 5 µl of lysate (i.e., DNA template), 12.5 µl of Absolute SYBR Green PCR mix (ABgene, Rochester, NY; contains the buffer, $MgCl_2$, dNTPs, and DNA polymerase), and the individual array of primers designed for use in this bacteriocin PCR array. The final concentration of primers used was 50 nM and the final reaction volume was 25 µl. The reaction mix was placed in PCR reaction tubes [MJ white low profile tubes (MJ Research, Hercules, CA)] and then subjected to real time PCR detection using the Opticon-2 DNA engine (MJ Research) with the following cycling profiles: initial denaturation at 95 °C for 15 min (genome denaturation), followed by 40 cycles of 95 °C for 15 s (denaturation), 62 °C for 60 s (annealing), 72 °C for 60 s (extension), followed by a final hold at 4 °C. All PCR runs included a blank control consisting of PCR-grade water and a non-template control (no DNA) which was run in parallel to determine amplification efficiency within each experiment. At the end of each run a melting curve analysis was performed from 58 °C to 90 °C at 0.2 °C/s to confirm the specificity of amplification and demonstrate the lack of primer dimer formation. Real-time PCR reactions were complemented by agarose gel electrophoresis, melting curve analysis, and DNA sequence analysis.

2.4. DNA sequencing and sequence analysis

Bacteriocin sequence information was analyzed by using the BLAST algorithm through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast). DNA sequencing of amplified DNA was performed at the Dept. of Biochemistry and Molecular Biology Recombinant DNA/Protein Resource Facility (Oklahoma State University) using an automated DNA sequencer via "BigDye™"-terminated reactions analyzed on an ABI Model 3700 DNA Analyzer. Bacteriocin gene sequences were compiled from DNA sequence reactions performed in both directions. Mega4 sequence analysis software (Tamura et al., 2007) was used to read ABI sequence files in which automatically generated sequence data was manually examined and compared to laser-dye-generated chromatograms to eliminate questionable base selections. Nucleotide multiple sequence alignment (MSA) was performed using the BCM Search Launcher online utility from the Baylor College of Medicine (Smith et al., 1996).

3. Results

3.1. PCR analysis of bacteriocinogenic LAB using a PCR bacteriocin primer array

We examined bacteriocinogenic LAB isolated from retail foods (Table 1; Garver and Muriana, 1993; Macwana and Muriana, 2012) by