Cloning and Sequencing of the Gene Encoding Curvaticin FS47, an Anti-Listerial Bacteriocin Produced by *Lactobacillus curvatus* FS47

S. Macwana, L. Ma, M.A. Cousin, and P.M. Muriana

Story in Brief

Curvaticin FS47 is a heat stable bacteriocin produced by *Lactobacillus curvatus* FS47. This bacteriocin inhibits the growth of the foodborne pathogen *L. monocytogenes* and other bacteria found in food. Curvaticin FS47 has been proposed as a potential "biopreservative" for use in food. The current research is done to provide a better understanding of the genetic determinants for curvaticin FS47. A biotinylated 29-mer oligonucleotide probe was designed from the N-terminal amino acid sequence of curvaticin FS47 and used to identify the location of the structural gene. Southern blot analysis indicated the presence of a 10kb bacteriocin operon. Several large chromosomal fragments obtained by the digestion with several enzymes were cloned into pBluescript. Using these clones, the nucleotide sequence for about 6kb of the bacteriocin was determined. The structural gene *(crvA*) was sequenced. Sequence analysis revealed nine open reading frames (ORFs) organized into three operon like structures. The structural gene, *crv*A, is located in a putative *crv* operon that consists of six ORFs. The peptides encoded by crvB and crvC in the crvA operon exhibited strong similarity to Class II LAB bacteriocins. This information will provide a basis for genetic manipulations and improve bacteriocin functionality in food-grade lactic acid bacteria.

Key Words: Bacteriocin, *L. curvatus*, Operon, Food Preservative

Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a number of different bacteria. Gram positive Lactic Acid Bacteria (LAB) have attracted attention for potential use as food preservatives. Bacteriocins are heat- stable inhibitory peptides. Nisin, a bacteriocin secreted by *Lactococcus lactis*, is commercially used as a food preservative. A wider use of bacteriocins as antimicrobial agents in milk and diary foods is hindered by the low activity of many bacteriocins in food.

Bacteriocins of LAB can be classified into four distinct classes based on biochemical and functional properties (Klaenhammer,1993). Class I or Lantibiotics are bacteriocins characterized by the presence of unusual amino acids such as lanthionine. Class II are small, heat stable non-lanthionine peptides. 3 subclasses:Class IIA are *Listeria*-active peptides, Class IIB are poration complexes with two peptides involved in their activity. Class IIC are Thiol-activated peptides where reduced cysteine residues are required for activity. Class III are large heat-labile proteins (M.W.>30 kDa). Class IV bacteriocins are complex compounds in which chemical moieties such as carbohydrates and lipids are required for activity.

Lactobacillus curvatus is a psychrotroph which can grow at temperatures as low as 5°C is known for the production of several different bacteriocins (curvacin, curvaticin) that are inhibitory to *Listeria monocytogenes* and some spoilage microorganisms. Amino acid sequence analysis of

curvacin A has shown that it shares N-terminal sequence homology with bacteriocins of other bacterial genera within the lactic acid bacteria group. Curvaticin FS47 is a listeria-active, heat stable bacteriocin produced by *L. curvatus* FS47 that was isolated from retail meats (Garver and Muriana, 1994). Although curvaticin FS47 does not contain the consensus sequence shared by Class IIa Listeria-active peptides, it inhibits the growth of *L. monocytogenes* suggesting that this must be done by a different mechanism.

Materials and Methods

Bacterial Strains, Plasmids, and Media. *Lactobacillus* strains were maintained as frozen stocks held at -20^{o}C in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) plus 10% glycerin (Fisher Scientific Co., Raleigh, NC). *Lactobacillus* cultures were grown in MRS broth or on MRS agar (1.5% agar) at 37^{o}C. Indicator overlay agar was prepared by adding 0.75% agar to MRS broth. Frozen stocks of *Escherichia coli* strains were maintained in Luria-Burtani (LB) broth plus 10% glycerin. *E. coli* DH5α was grown in LB broth at 37^{o}C with shaking (240 rpm). *E. coli* DH5α cells transformed with pBSKS(+) and its derivatives were grown in LB broth containing ampicillin (Amp) at a final concentration of 100 ug/ml, or on selective LB agar (100ug/ml Amp; 0.5 mM IPTG; and 40 ug/ml X-gal).

DNA Isolation and Manipulation. Isolation of plasmid DNA from *Lactobacillus* strains was performed by the method of O'Sullivan and Klaenhammer (1993). Plasmid DNA from *E.coli* strains was prepared by alkaline lysis. Total DNA of *L. curvatus* strains was isolated by the procedure of Wu and Muriana (1995). Plasmid DNA required for sequencing was further purified by CsCl-ethidium bromide density gradient ultracentrifugation. Electric-shock transformation (electroporation) of *E. coli* DH5α with plasmid DNA was done using a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, CA) according to instructions from the manufacturer.

Genomic Clones. A biotinylated 28-mer oligo probe determined from the protein sequence of curvaticin FS47 was used to obtain two overlapping clones in pBluescript KS(+) encompassing the curvaticin FS47 structural gene, *crv*A. Smaller fragments were subcloned from these larger genomic clones, purified, and submitted for sequencing. Sequencing of plasmid clones, subclones, or PCR products was obtained through either the Purdue University Core Sequencing facility or the Oklahoma State University DNA Core Facility.

Cloning and Subcloning. DNA fragments of the appropriate size range were electro-eluted from bands excised from 0.8% agarose gels and ligated to pBSKS+ that was linearized with the same enzyme. The ligation mixture was then electroporated into *E. coli* DH5α cells using the Bio-Rad gene pulser. Colonies containing potential pBSKS+ recombinant plamids were identified by the blue-white color reaction on X-gal.

Sequence Analysis. Open reading frames (ORFs) were determined using the ORF Finder provided online at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) and BLAST was used to determine protein sequence similarity.

Differentiation of *L. sakei* and *L. curvatus*. PCR amplification: Species-specific primer combinations have been identified to differentiate *Lactobacillus curvatus* from the related *Lactobacillus sakei* which involves a primer for the common sequence of the 16S rRNA gene of both and another for a species-specific spacer region. Amplification consisted of 20 cycles (for primer pairs 16/Ls, 16/Lc) of: 1min at 94^{o}C, 30 sec at 45^{o}C and 1min at 72^{o}C. The first cycle was incubated for 5 min at 94^{o}C. A 10 ul aliquot of the PCR products were electrophoresed in a 1% agarose gel and visualized by UV illumination after ethidium bromide staining.

Results and Discussion

Identifying the Bacteriocin Producing Species. For species-specific PCR amplification, each species-specific primer was paired with the forward primer from the 16s rRNA gene. As seen in Fig.1 conditions that allowed the amplification of a PCR fragment from DNA occurred only in the presence of the expected pair of primers.

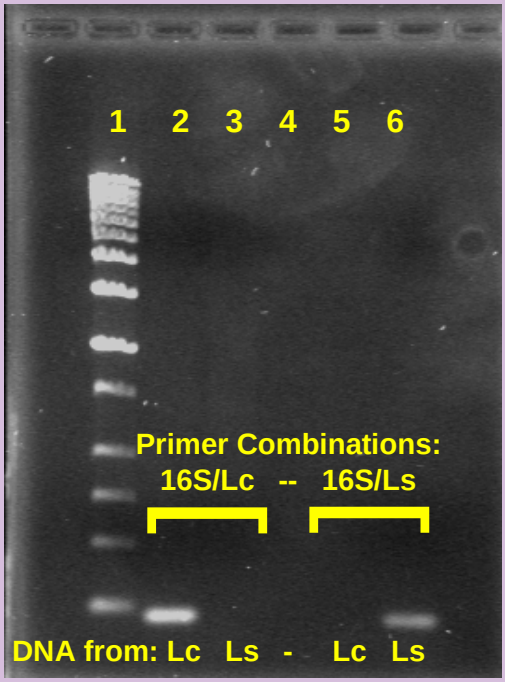

Figure 1. PCR amplification of species-specific DNA targets to help identify the bacteriocin-producing strain FS47. A combination of 16S rRNA-specific primers and primers for the 16S/23S rRNA spacer regions specific for either Lactobacillus curvatus (16S/Lc) or for *Lactobacillus sakei* (16S/Ls). *L. curvatus* FS47 (lanes 2, 5); *L. sakei* 790 (lanes 3, 6); 1-kb DNA ladder (lane 1).

Detection of the curvaticin FS47 structural gene (crvA). The gross location of the curvaticin FS47 structural gene, *crvA*, was determined from Southern blot analyses with uncut plasmid/chromosomal DNA and with restriction enzyme-digested DNA from *L. curvatus* FS47 and other *L. curvatus* strains. No signal was obtained from plasmid DNA in Southern blot analyses, whereas strong hybridization signals were obtained from uncut chromosomal DNA (Fig. 2A). These data demonstrate that the structural gene of curvaticin FS47 is located on the chromosome. Subsequent Southern blot analyses with restriction-digested chromosomal DNA

were performed to identify intermediate-sized fragments after digestion that would be useful sizes for cloning (i.e., large enough to likely contain several or more genes) (Fig. 2B).

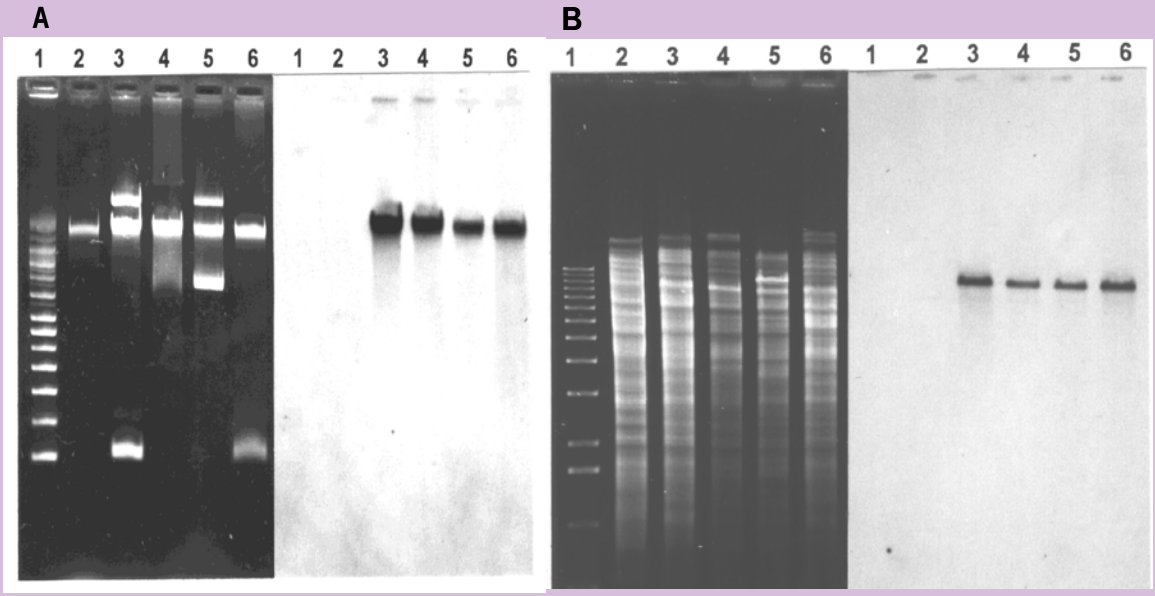

Figure 2. Agarose gel (dark panels) and Southern hybridization analysis (light panels) of un-digested plasmid/chromsomal DNA (Panel A) and EcoRI-digested total genomic DNA (Panel B) from *L. curvatus* strains using a biotinylated 28-mer nucleotide probe. Lanes: 1, Supercoiled (Panel A) or linear (Panel B) DNA standards; 2, *L. curvatus* ATCC 25601 (Bac-); 3, *L. curvatus* FS65 (Bac+); 4, *L. curvatus* FS47 (Bac+); 5, *L. curvatus* FS44 (Bac+); 6, *L. curvatus* FS36 (Bac+).

Sequencing of Various Clones and Subclones. Initial sequencing of clones led to the identification of a 4,379 bp of sequence information. Nine open reading frames were identified among three operon-like structures that were transcribed in different directions. One operon-like structure yielded contained the *crv*A structural gene (Fig. 3). To date, approximately 8,173 bp of nucleotide sequence has been sequenced surrounding the *crv*A structural gene (data not shown).

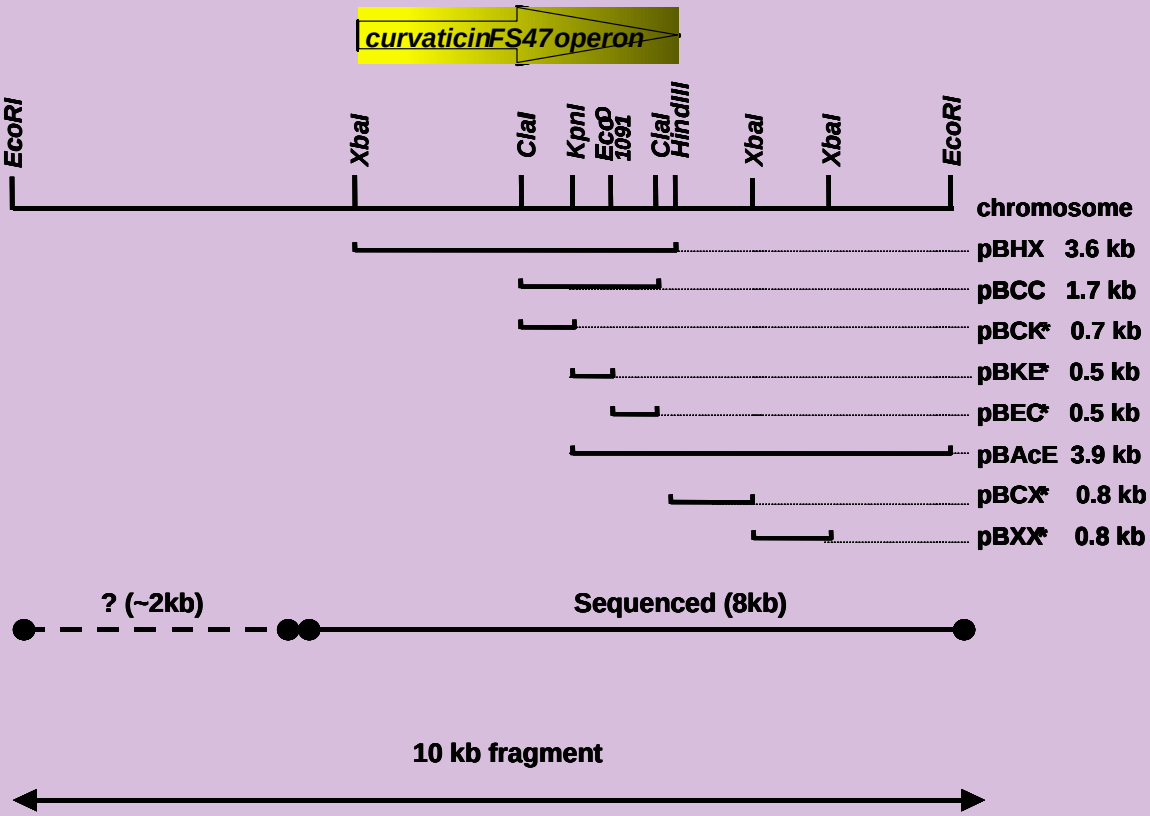

Figure 3. Schematic of restriction map of *L. curvatus* FS47 chromosomal DNA surrounding the *crv*A region. Only restriction enzyme sites relevant for the construction of the various subclones are shown. The sizes of the cloned fragments are indicated on the right. Asterisks represent subclones used for nucleotide sequencing.

The *Lactobacillus curvatus* FS47 bacteriocin operon is nearly completely sequenced and show a high degree of homology to a bacteriocin from *L. sakei*, yet there is a stretch of sequence outside this region that is different as PCR primers based on *L. sakei* did not amplify DNA sequence from *L. curvatus*. This research still awaits the potential use of this bacteriocin as an anti-listerial food preservative.

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Author List

Cousin, Maribeth. Professor, Department of Food Science, Purdue University

Ma, Li. Ph.D. student, Department of Food Science, Purdue University

Macwana, Sunita. Ph.D. student, Department of Animal Science, Oklahoma State University

Muriana, Peter. Associate Professor, Department of Animal Science & Food and Ag Products Center, Oklahoma State University