A Novel Approach for Rapid Identification and Sequencing of Different Bacteriocins Produced by LAB Based on a Practical 'Immunity Class'

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Story in Brief

Bacteriocins (i.e., inhibitory proteins) produced by lactic acid bacteria (LAB) are considered "natural antimicrobials" that may enhance the safety of foods. With numerous claims of "new bacteriocins", it is important to employ methods to identify or distinguish bacteriocins that have not been previously characterized.

Bacteriocin-resistant (BacR) strains of *L. monocytogenes* were obtained by selection against LAB bacteriocins. The BacR *Listeria* was then used as indicators to find additional bacteriocin-producing strains from retail foods. This cycle was repeated three consecutive times resulting in the aggregate accumulation of resistance in the same strain to three different classes of bacteriocins based on immunity. Bacteriocin-producing strains of interest were then subjected to a SYBR Green-based real-time PCR array using sets of primers made from the coding sequence for all known LAB bacteriocin structural genes and sequenced at our university core facility.

The method described allows us to identify different functional 'immunity classes' based on bacteriocin resistance screening and a PCR array provided quick amplification and sequence identity of the various structural genes to determine if these bacteriocins were previously studied.

Keywords: Real Time PCR, Fluorescence, Screening, Bacteriocins

Introduction

Over the last three decades, a variety of lactic acid bacteria that are generally-recognized-as-safe (GRAS) for use in foods have been found to produce bacteriocins that kill or inhibit the growth of other bacteria. Because of potential food-related applications, many bacteriocins have been identified and characterized, both biochemically and genetically.

Consumers have been concerned about possible adverse health effects from the presence of chemical additives in their foods. As a result, consumers are often drawn to natural and "fresher" foods with no chemical preservatives added. This perception, coupled with the increasing demand for minimally-processed foods with long shelf life and convenience, has stimulated research interest in finding natural but effective preservatives. Bacteriocins, produced by LAB, may be considered natural preservatives or biopreservatives that can possibly fulfill these requirements. Biopreservation refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in foods to enhance food safety and extend shelf life.

The traditional cultural and molecular techniques for the isolation, purification, and sequencing of bacteriocin peptides, and further cloning of bacteriocin-related genes is very laborious and time-consuming and to the dismay of the investigator, may result in the identification of bacteriocin sequences already residing in GenBank (i.e., from work of prior investigators). A method that would facilitate the quick practical and sequence-identity of such bacteriocins of interest would be most desirable in this heavily studied field.

Our study combines two processes to a) differentiate groupings of bacteriocins based on ‘immunity class’ and b) using a PCR array of primers for all known bacteriocin structural genes of LAB to quickly amplify the gene sequence and submit them for sequencing to compare with what currently resides in the GenBank database. An ‘immunity class’ may be considered to exist when selection of a BacR derivative provides resistance to a particular bacteriocin; another bacteriocin which may still inhibit the BacR strain would be considered to belong to a different ‘immunity class’. The compilation of bacteriocins of different immunity classes could have practical implications in the development of an anticmicrobial bacteriocin ‘cocktail’ against which the development of resistance would not readily occur. The combination of these two processes allows for the quick and facile discovery and sequence identity of functionally different and novel bacteriocins.

Materials and Methods

Bacteriocin Testing. Screening of bacteriocin-producing cultures from foods or culture collections were described previously (Muriana and Luchansky, 1998; Muriana, 1996). Spent cultures of various bacteriocin-producing LAB were adjusted to pH 7.0 and filter-sterilized (0.2 µ) before use in the inhibitory spot test on indicator lawns.

Derivation of Bacteriocin-resistant Strains. Isolated colonies of the *L. monocytogenes* indicator strain that would appear within inhibitory zones upon extended incubation were re-streaked on bacteriocin containing agar and in subsequent lawn assays were found to be resistant to the bacteriocin. A bacteriocin-resistant strain could be made resistant to several different bacteriocins by applying this same method in succession.

Primer Design. All coding strand sequences for known LAB bacteriocin structural genes were retrieved from the GenBank Database. Primers were designed using Vector NTI (ver. 9.0) software. A total of 36 pairs of primers were designed from the coding strands for all sequenced bacteriocin structural genes targeting *Lactobacillus, Pediococcus, Lactococcus*, and *Leuconostoc* species.

Screening by Real-time PCR. Cultures used as targets for real time PCR were transferred twice and grown overnight in MRS broth at 30°C. Prior to PCR, the DNA for all Gram-positive strains used in this study was extracted by the BAXT^{m} procedure (Qualicon). Absolute SYBR Green PCR mix was used according to manufacturer’s instructions with the primers designed in this study. To this reaction mix real-time PCR was performed using the Opticon-2 DNA engine (MJR research); cycling profiles: initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s (denaturation), 62°C for 60 s (annealing), 72°C for 60 s (extension), and a final hold at 4°C.

Results and Discussion

Listeria monocytogenes is inhibited by numerous bacteriocins produced by Lactic Acid Bacteria (LAB). Selection for bacteriocin-resistant (BacR) variants of *L. monocytogenes* resulted in strains that were resistant to one or more bacteriocins and demonstrated that resistance to one bacteriocin can provide cross-resistance to others. This prompted the notion of grouping bacteriocins based on ‘immunity classes.’ Using BacR strains of *L. monocytogenes* that were successively resistant to different bacteriocins, we were able to identify bacteriocins that were unaffected by resistance developed towards other bacteriocins. We believe the use of mixtures of bacteriocins of different immunity classes will

improve the use of bacteriocins as ‘biopreservatives’ in regard to minimizing or eliminating the appearance of BacR variants when used in food applications.

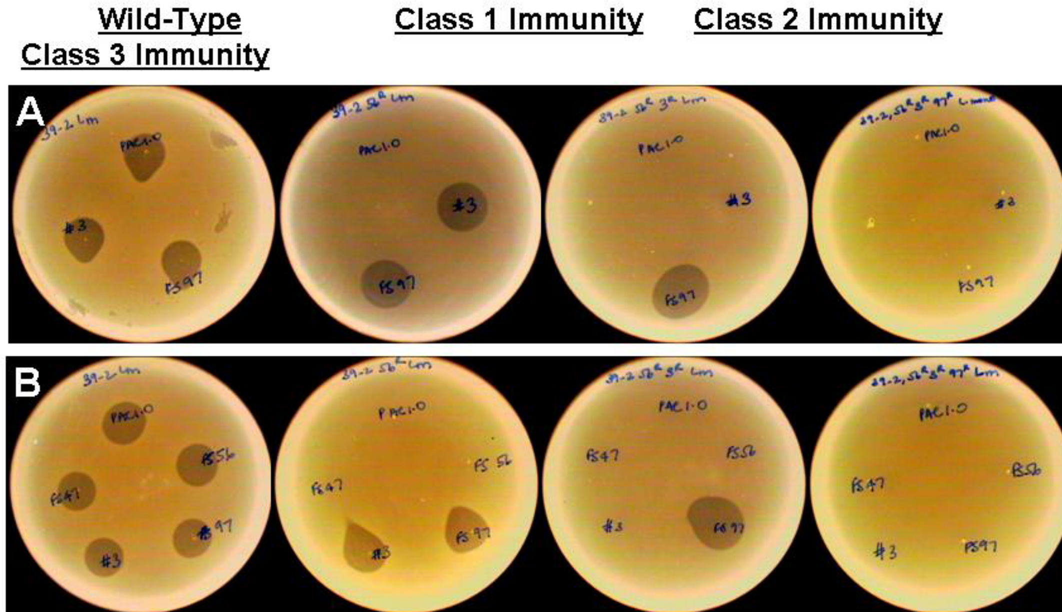

Figure 1. Inhibitory zones of filter-sterilized, pH-adjusted (pH 7.0) bacteriocin extracts on various wild-type and bacteriocin-resistant indicator lawns (as listed above the plates). Panel A, bacteriocin extracts from *Pediococcus acidilactici* PAC1.0, *Lactococcus lactis* FS97, and Bacteriocin Isolate #3 spotted on the wild-type *Listeria monocytogenes* 39-2, and strain 39-2 that was resistant to the *P. acidilactici* PAC1.0 bacteriocin, strain 39-2 that was resistant to 2 bacteriocins (PAC1.0 and Bacteriocin#3), and strain 39-2 that was resistant to all 3 bacteriocins. Panel B, bacteriocin inhibitory zones using 5 bacteriocins: 3 Class 1 immunity bacteriocins (*Lactobacillus curvatus* FS47, *Pediococcus acidilactici* PAC1.0, *Lactococcus lactis* FS56), a Class 2 immunity bacteriocin (Bacteriocin Isolate #3), and a Class 3 immunity bacteriocin (*L. lactis* FS97).

Bacteriocin producing strains Bact #3 and *L. lactis* FS 97, along with other bacteriocins, were identified by our screening process using the various BacR *L. monocytogenes* indicator strains. These strains were then subjected to a SYBR Green-based real-time PCR array using sets of primers made from the coding sequence for all known LAB bacteriocin structural genes and the resulting amplimers were sequenced at our OSU core facility.

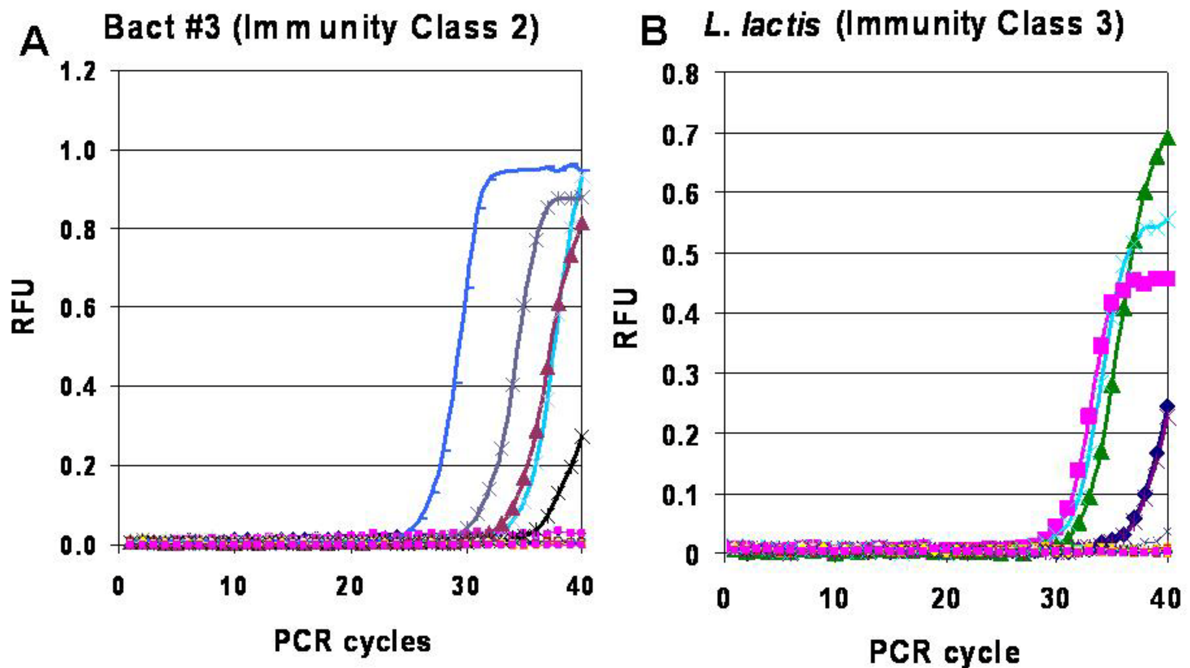

Figure 2. Real-time PCR amplification using the PCR array of LAB bacteriocin structural genes. Panel A, SYBR green amplification of a Bac+ strain (Bact #3) of immunity class 2 from ground turkey sample. Panel B, SYBR green amplification of a Bac+ immunity class 3, *L. lactis* FS97 as template. Both panels include non-template controls.

During primer design, it was obvious that the high homology exhibited by the numerous bacteriocin structural genes would make it difficult for a discrete primer set to be made to amplify only a specific bacteriocin. We therefore simplified the PCR array by using a primer set based on maximizing the PCR product from each bacteriocin without regard to homologous primer target sequences and knowing that a particular bacteriocin strucutural gene may be amplified by more than one pair of primers from our array. We figured that the sequence of the internal amplimer, however, would be specific to the particular bacteriocin that was used for template DNA.

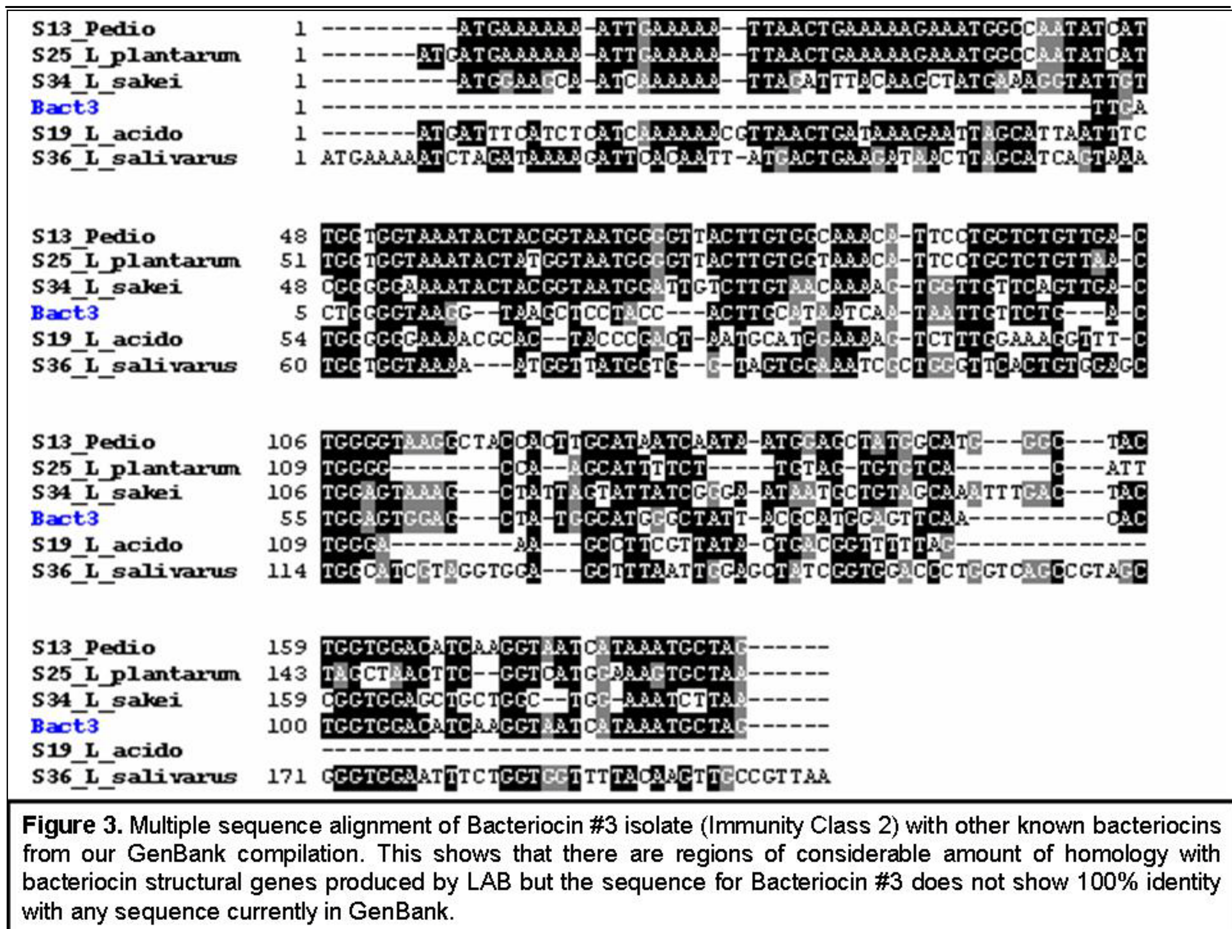

Figure 3. Multiple sequence alignment of Bacteriocin #3 isolate (Immunity Class 2) with other known bacteriocins from our GenBank compilation. This shows that there are regions of considerable amount of homology with bacteriocin structural genes produced by LAB but the sequence for Bacteriocin #3 does not show 100% identity with any sequence currently in GenBank.

This approach for both bacteriocin selection and PCR amplification with sequencing followed by GenBank database comparison allowed for quick identification of new and novel bacteriocins.

Literature Cited

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