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Full Paper

Screening and characterisation of bacteriocin-producing bacteria capable of inhibiting the growth of bovine mastitis

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Abstract: A total of 302 bacterial strains were isolated from 43 raw milk samples collected from dairy farms in Chiang Mai province. Out of these strains isolated, three strains were found to produce bacteriocins against *Streptococcus dysgalactiae* DMST10953, a bovine mastitis pathogen. These were *Lactobacillus fermentum* RMM701, *Streptococcus bovis* RMM703 and *Streptococcus bovis* RMM902, which exhibited bacteriocin activity at 40, 40 and 20 AU/ml respectively. Bacteriocins produced by these three strains were heat-stable up to 80 ^{o}C. Bacteriocins produced by *L. fermentum* RMM701 and *S. bovis* RMM902 were stable at pH 2.0 - 7.0 while that from *S. bovis* RMM703 was stable at pH 2.0 - 6.0. These bacteriocins were also found to be inactivated by proteolytic enzymes such as proteinase K and subtilisin A.

Keywords: bacteriocin, bovine mastitis, *Streptococcus dysgalactiae*

Introduction

Bovine mastitis is an inflammation of the mammary glands usually due to a microbial infection that affects milk production and quality, being one of the most significant causes of economic loss to the dairy industry [1]. *Staphylococcus aureus, Streptococcus dysgalactiae* and *Streptococcus uberis* are still the dominant pathogens [2]. Bovine mastitis is usually treated or prevented with intramammary

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antibiotics [3,4]. Although the use of antibiotics to control mastitis is normally very effective, it has some disadvantages, including the appearance of residues in the milk of treated cows [5] and the perceived connection to the emergence of antibiotic-resistant human pathogens, particularly the increased incidence of organisms such as methicillin-resistant *St. aureus*, which is prevalent in nosocomial infections in humans [6]. Thus the identification of alternative methods for controlling this illness is essential. One of these methods could be the use of bacteriocin.

Bacteriocins are proteinaceous antimicrobial agents and inhibit or kill closely related species of bacteria [7]. Some bacteriocins inhibit food spoilage and pathogenic microorganisms. They are therefore potentially useful as natural replacements for synthetic food preservatives or used in such medical and veterinary applications as the prevention and treatment of some infectious diseases [8,9].

In a previous study, nisin produced by *Lactococcus lactis* is effective against a wide range of Gram-positive bacteria, including mastitic pathogens such as *St. aureus* ATCC 2970, *S. dysgalactiae* ATCC 27957, and *S. uberis* ATCC 27958 [10]. In addition, when teat seal was blended with lacticin 3147 produced by *Lc. lactis* and infused into teats of nonlactating cows, the formulation reduced the incidence of clinical mastitis in teats that had been inoculated with *S. dysgalactiae* from 42% in control quarter to 6% in treated quarters [11].

The aims of this study are to screen bacteriocin-producing bacteria capable of inhibiting the growth of bovine mastitis pathogens and to study the physicochemical characteristics of these bacteriocins.

Materials and Methods

Bacterial strains and culture conditions

Three hundred and two bacterial strains were isolated from 43 raw milk samples in Chiang Mai province and used in the screening for bacteriocin production. *St. aureus* RMB203 and *St. aureus* RMB1601, were isolated from bovine mastitis. *S. dysgalactiae* DMST 10953 and *S. agalactiae* DMST 11366 were used as indicators. All lactic acid bacteria were cultivated in De Man, Rogosa and Sharpe (MRS) medium and incubated anaerobically at 37 ^{o}C for 16 h in anaerobic jars having a H_2+CO_2 environment generated with a BBL GasPak (Becton Dickinson Microbiology systems). Other non-lactic acid bacteria were grown in Brain Heart Infusion (BHI) medium at 37 ^{o}C for 16 h under aerobic condition. The bacteria were stored at -80 ^{o}C in 20% glycerol until needed.

Initial screening of bacteriocin-producing bacteria

The bacteriocin-producing bacteria were initially screened by spot agar test [12]. A total of 302 bacterial strains were grown in their appropriate culture medium and conditions: lactic acid bacteria were cultivated in MRS medium and incubated anaerobically at 37 ^{o}C for 16 h, and other non-lactic acid bacteria were grown in BHI medium at 37 ^{o}C for 16 h under aerobic condition. These bacteria was spotted onto BHI agar plate. After 16 h of incubation at 37 ^{o}C in aerobic condition, the plates were

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overlayed with soft BHI agar (0.7 % agar) containing indicator cultures. The plates were incubated overnight at 37 ^{o}C to assess the activity of these bacteria against each indicator strain.

Preparation of bacteriocin samples

Bacterial strains were cultivated in their appropriate culture medium and conditions. Extraction of bacteriocin was carried out using the method of Schillinger and Lucke [12]. Cells were removed by centrifugation at 5,000 rpm for 10 minutes at 4 ^{o}C. The supernatant were adjusted to pH 6.5 with 5M NaOH and 5M HCl to exclude the antimicrobial effect of organic acids. Inhibitory activity from hydrogen peroxide was eliminated by the addition of 1 mg/ml catalase, followed by filtration of the supernatant through a 0.2 µm pore-size nylon syringe filter. Supernatants were stored at -20 ^{o}C.

Bacteriocin bioassay

Antimicrobial activity against indicator organisms was determined using a well diffusion assay [12]. Pre-poured BHI agar plates were overlaid with BHI soft agar containing indicator cultures. Wells of 6 mm in diameter were cut into the agar plate with a cork borer and 50 µl of the cultured supernatant fluid was placed into each well. The plates were incubated overnight at 37 ^{o}C. The antimicrobial activity of the bacteriocin is defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and is expressed in activity units per ml (AU/ml).

Identification of bacterial strains

Biochemical characteristics: Bacterial strains were cultivated in MRS medium and incubated anaerobically at 37 ^{o}C for 16 h. Sugar fermentation reactions were recorded by using the API 50 CH and API 20 STREP (BioMérieux).

Extraction, amplification and sequencing of bacterial DNA: DNA extraction was conducted by using the commercial Isoplant DNA extraction kit (NO. 314-02731, Nippon Gene, Japan) with the manufacturer's protocol. Extracted DNA was stored at -20 °C until needed. The 16S rDNA was amplified by polymerase chain reaction (PCR) using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 520R (5'-ACCGCGGCKGCTGGC-3') (Operon, Germany). PCR was performed in a PCR Sprint Thermal Cycler (Sprint, Thermal hybrid). For each reaction, a 50-μl reaction mixture was prepared. It consisted of 25 μl of Qiagen master mix, 2 μl of 27F primer, 2 μl of 520R primer, 20 μl of sterile H_2O, and 1 μl of 20 ng/ μl bacterial DNA. The amplification was programmed as follows: initial denaturation at 94 ^{o}C for 5 min, followed by 25 cycles at 94 ^{o}C for 1 min, 55 ^{o}C for 1 min, and 72 ^{o}C for 1 min. After these cycles, the reaction was maintained at 72 ^{o}C for 5 min and then cooled to 4 ^{o}C. Five microlitres of the PCR products were visualised after electrophoresis in a 1.5 % agarose gel and were subsequently visualised by UV illumination after ethidium bromide staining. The PCR products were purified by using the TaKaRa SUPREC^{TM}-PCR (Takara, Japan) according to the manufacturer's instructions, and analysis of all sequences was performed by First Base Laboratories Company (Malaysia).

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Sequence data analysis: All of the sequencing data were analysed with the BLAST (The National Centre for Biotechnology Information; NCBI).

Characterisation of bacteriocin

The bacteriocin samples were characterised with respect to heat, pH stability and susceptibility to denaturation by enzymes.

Heat resistance: Samples of bacteriocin were exposed to various heat treatments: 40, 60, 80, 100 and 121ºC. Aliquot volumes were then removed after 0, 30, 60 and 90 minutes [13] and assayed for bacteriocin activity.

pH sensitivity: Samples of bacteriocin were adjusted to pH 2, 4, 6, 8, 10, and 12 with 5N HCl or 5N NaOH. After incubating for 4 h at room temperature the bacteriocin samples were adjusted to pH 6.5 [13] and assayed for bacteriocin activity.

Enzyme treatment: The sensitivity of the bacteriocin to different enzymes was checked. The cell-free supernatant fluid at pH 6.5 was treated separately with protease and proteinase K at a final concentration of 1.0 mg/ml. Samples of bacteriocin were incubated with each enzyme for 2 h at 37 ºC. After that samples of bacteriocin were heated for 5 minutes at 100 ^{o}C to inactivate enzyme activity [14] and assayed for bacteriocin activity.

Results and Discussion

Isolation of bacteria and screening of bacteriocin producing bacteria

A total of 302 bacterial strains were isolated from 43 raw milk samples in Chiang Mai province. These isolates were tested for antimicrobial activity against 4 indicator strains. Only 148 strains showed inhibition zones when they were analysed by the spot agar test. Out of these strains, only three strains (RMM701, RMM703 and RMM902) produced bacteriocins against *S. dysgalactiae* DMST 10953, which is a bovine mastitis pathogen, when cell-free supernatants were analysed by the well-diffusion assay. Figure 1 shows the sizes of the inhibition zone against *S. dysgalactiae* when 10% (v/v) lactic acid was used as positive control. Bacteriocins produced by these three strains exhibited bacteriocin activity at 40, 40 and 20 AU/ml respectively (Table 1).

Table 1. Activity of bacteriocin produced by three bacterial strains against *Streptococcus dysgalactiae* DMST 10953

Strain	Inhibition zone (mm)*	Bacteriocin activity (AU/ml)
Positive control	12.4 $\pm$ 0.6	-
RMM701	9.3 $\pm$ 0.6	40
RMM703	9.7 $\pm$ 0.6	40
RMM902	9.7 $\pm$ 0.6	20

* well = 6.0 mm

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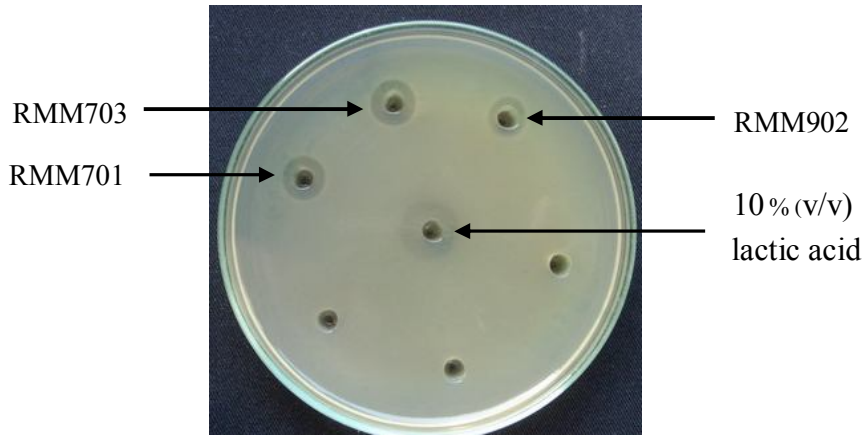

Figure 1. Results of the well-diffusion assay of three bacterial strains which produced bacteriocins against *Streptococcus dysgalactiae* DMST10953. (The rest of three wells showed negative results.)

Identification of bacterial strains

Strains RMM701, RMM703 and RMM902 were identified as *Lactobacillus fermentum*, *Streptococcus bovis* and *Streptococcus bovis* by 75.8, 97.6 and 97.6% homology respectively, based on API 50 CH and API 20 STREP (BioMérieux) profiles (not shown) and their physiological characteristics. The 16S rDNA was amplified from three bacterial strains. Fragments of about 500 bp were obtained (Figure 2.). The 16S rDNA sequence of RMM701, RMM703 and RMM902 were determined and compared with available 16S rDNA sequences in the Genbank database. The sequences were similar to *L. fermentum* RMM701, *S. bovis* RMM703 and *S. bovis* RMM902 by 99.1, 98.9 and 99.4 % homology respectively (Table 2). Thus, these strains were chosen for further characterisation.

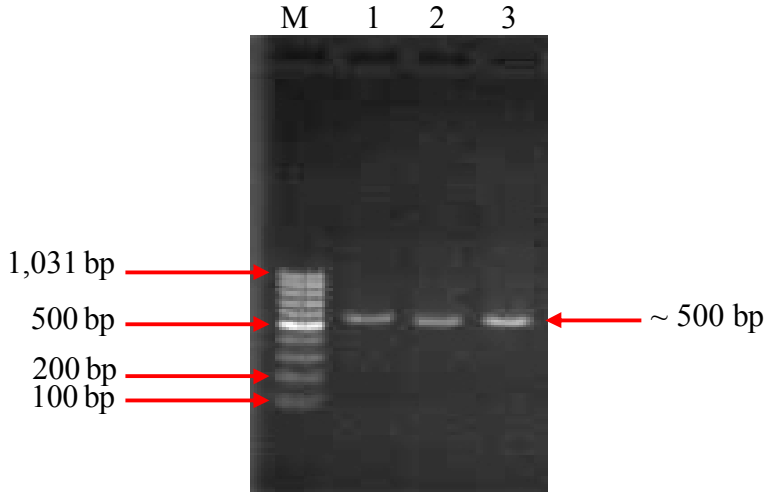

Figure 2. Ethidium bromide-stained agarose gel electrophoresis of amplified 16S rDNA of bacterial strains. Lane M: 100 bp DNA ladder used as molecular size marker; lane 1: RMM701; lane 2: RMM703; lane 3: RMM902.

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Table 2. Identification of bacteriocin-producing bacteria by 16S rDNA sequencing*

Bacterial strain	Bacteria	Accession number	Identity	% Homology
RMM701	*Lactobacillus fermentum*	DQ523484.1	522/527	99.1
RMM703	*Streptococcus bovis*	AB186306.1	525/531	98.9
RMM902	*Streptococcus bovis*	DQ467871.1	482/485	99.4

* Analysis date : 10 August 2007

Effects of temperature, pH and enzymes on the bacteriocin activity

The effects of heat, pH and enzymes on bacteriocin activity were determined using *S. dysgalactiae* DMST 10953 as indicator organism. Bacteriocins produced by the three strains (RMM701, RMM703 and RMM 902) were heat-stable at 80^{o}C for 60 min although their activities were reduced twofold. At 100 ^{o}C there was no detectable bacteriocin activity in these three strains (Figure 3). Heat sensitivity of these bacteriocins may be due to their non-complex, linear structures. Similar results of heat sensitivity were recorded for bozacin 14 [15]. Most bacteriocins were heat stable at 121 ^{o}C such as pediocin P [16], lactocin LC-09 [17] and nisin Z [18]. The heat stability may be due to the formation of small globular structures and the occurrence of strongly hydrophobic regions, stable cross-linkages, and a high glycine content [19].

Bacteriocins produced by *Leuconostoc* sp. RMM701 and *S. bovis* RMM902 were stable at pH 2-7, while that of *S. bovis* RMM703 was stable at pH 2-6 (Table 3). The loss of activity at higher pH could be due to change of conformation of the molecule. This result was similar to the properties reported for bacteriocins produced by other lactic acid bacteria such as Pediocin AcH [20], Pediocin A [21], Lactacin [22], Nisin [23] and bovicin HC5 [24].

These bacteriocins were inactivated by proteolytic enzymes such as proteinase K and protease (Table 4). Inactivation of antimicrobial activity by protease and proteinase K suggested that the substances could be antimicrobial peptides [16].

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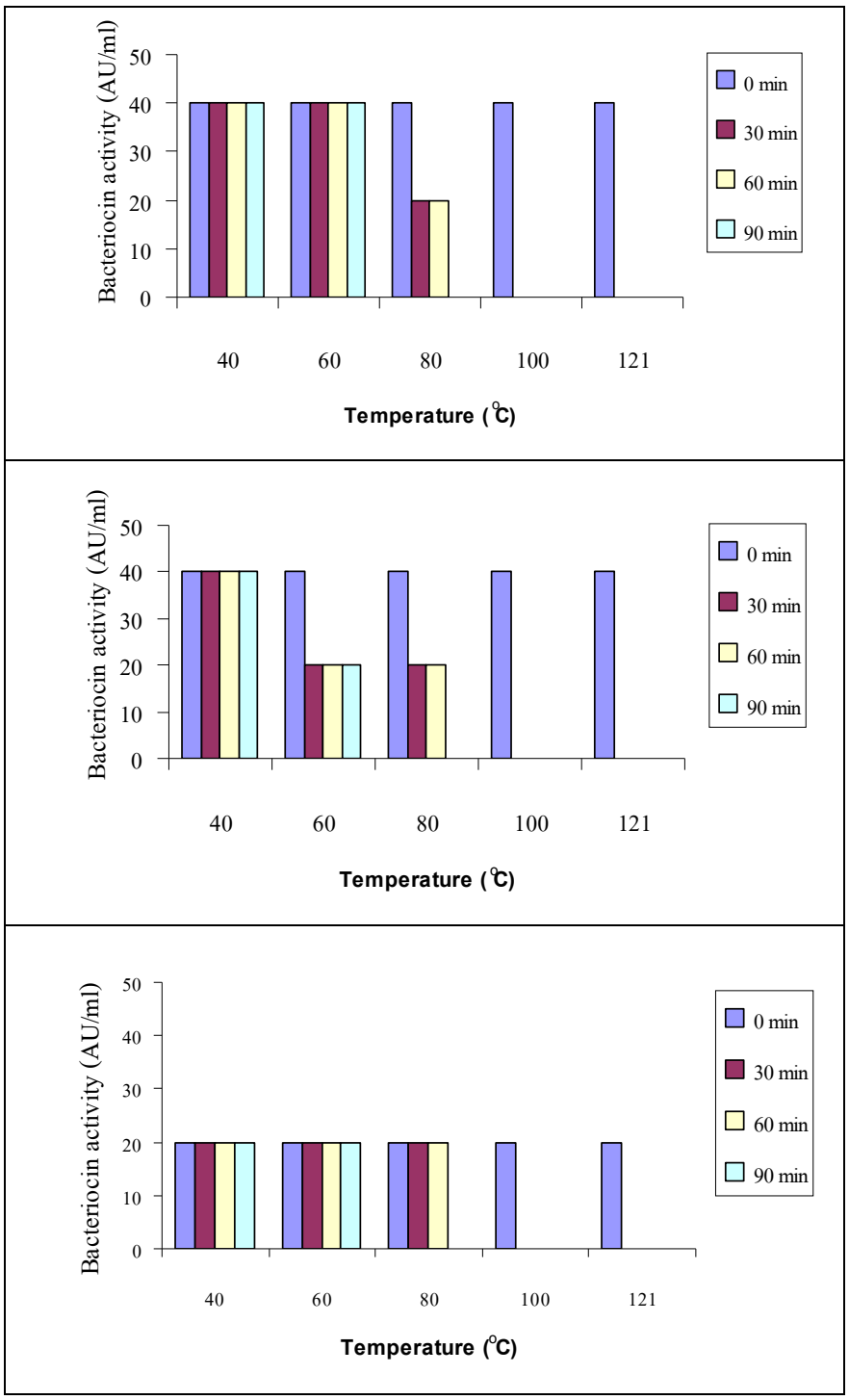

Figure 3. Effect of temperature on the activity of bacteriocin produced by each bacterial strain: (A) *L. fermentum* RMM701, (B) *S. bovis* RMM703, and (C) *S. bovis* RMM902

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Table 3. Effects of pH on activity of bacteriocin produced by each bacterial strain

pH	Bacteriocin activity (AU/ml)		
	L. fermentum RMM701	*S. bovis* RMM703	*S. bovis* RMM902
2	20	20	20
3	20	20	20
4	20	20	20
5	20	20	20
6	20	20	20
7	20	0	20
8	0	0	0
9	0	0	0
10	0	0	0

Table 4. Effects of enzyme on activity of bacteriocin produced by each bacterial strain

Enzyme	Bacteriocin activity (AU/ml)		
	L. fermentum RMM701	*S. bovis* RMM703	*S. bovis* RMM902
Control*	40	40	20
Proteinase K	0	0	0
Subtilisin A	0	0	0

* Crude extract of bacteriocins not treated with enzyme was used as control.

Conclusions

L. fermentum RMM701, *S. bovis* RMM703 and *S. bovis* RMM902 were isolated from raw milk samples from dairy farms in Chiang Mai province. They all produced bacteriocins against *Streptococcus dysgalactiae* DMST10953, a bovine mastitis pathogen. These bacteria exhibited bacteriocin activities at 40, 40 and 20 AU/ml respectively. Bacteriocins produced by these three strains were heat-stable up to 80 ^{o}C for 60 minutes, at which their activities were reduced twofold. Bacteriocins produced by *L. fermentum* RMM701 and *S. bovis* RMM902 were stable at pH 2.0 - 7.0 while that from *S. bovis* RMM703 was stable at pH 2.0 - 6.0. These bacteriocins were also found to be inactivated by proteolytic enzymes such as proteinase K and subtilisin A, suggesting that the substances could be antimicrobial peptides.

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