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A Novel Bacteriocin with Anti-MRSA Activity Produced by *B. amyloliquefaciens* Strain WARY9-1M and Its Characterization

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The number of methicillin resistant *Staphylococcus aureus* or MRSA infections has been increasing and become a serious problem in public heath worldwide. The use of bacteriocin as an alternative agent to overcome this problem is promising. The partially purified bacteriocin or PPB obtained from strain WARY9-1M showed broad antimicrobial activity.The most sensitive strains were MRSA and *S. aureus*. The PPB was heat stable up to 100°C for 60 min and active within the pH range of 3-9. The complete inactivation after treatment with all proteolytic enzymes (trypsin, α-chymotrypsin and proteinase K) reveals its proteinaceous nature. This isolated strain can be regarded as bacteriocin producing bacteria or BAC. It was identified as *Bacillus amyloliquefaciens* by 16S rRNA gene sequence. Tris-Tricine SDS-PAGE analysis revealed that bacteriocin from strain WARY9-1M had an apparent molecular weight of 3 kDa. PCR analysis showed the presence of malonyl CoA transacylase (*ituD*) and lichenysin synthetase C (*lchAC*). The novel bioactive peptide from *B. amyloliquefaciens* strain WARY9-1M has a potential for use as an alternative antibacterial agent for the treatment of infection with MRSA in the future.

Keywords: Bacteriocin, Anti-MRSA, *B. amyloliqefaciens*, WARY9-1M.

The emergence and dissemination of antibiotic resistance pathogenic bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) becomes an increasing serious problem in the public health worldwide[1]. Moreover, MRSA strains tend to accumulate additional new antibiotic resistance such as mupirocin[2]. New strategies for controlling MRSA and multiresistant staphylococci are urgently needed. Many substances used in pharmaceutical industries have been isolated from microorganisms. It is promising to use antibacterial compound known as bacteriocins to replace currently used antibiotics. Bacteriocins are ribosomally synthesized antimicrobial protein or peptide produced by bacteria that usually inhibit closely related species[3]. Current potential use of bacteriocins are in food industry as natural and safe food preservatives while less research has been conducted on the therapeutic applications as antimicrobial agent[4]. Bacteriocin production is common among many gram-positive bacteria[5] especially soil bacteria in the genus *Bacillus*. Like LAB, the genus *Bacillus* includes a variety of industrially species which has been granted Generally Recognized as Safe (GRSA) status by the Food and Drug Administration, USA[6]. In a previous research, strain WARY9-1M showing high antimicrobial activity against MRSA was isolated[7]. The objective of this study is to characterize the bacteriocin from bacteriocin-producing bacteria (BAC) strain WARY9-1M. This strain is not hazard and might be suitable for using as an alternative source of peptide antibiotic for control of many important antibiotic resistant pathogenic bacteria in the future.

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MATERIALS AND METHODS

Bacterial strain identification

The isolated strain WARY9-1M was identified according to its 16S rRNA gene sequence[8]. The nucleotide sequence was compared with genbank nucleotide database using Blastn search (http://www.ncbi.nlm.nih.gov/blastn) in order to identify the strain.

Determination of bacteriocin activity

The antibacterial activity of bacteriocin was detected by an agar-well diffusion method [9] and bacteriocin activity (AU/ml) was determined by the serial dilution method. The assay for each sample was done in triplicate.

Preparation of partially purified bacteriocin (PPB)

A 200 ml TSB medium was inoculated with 1% (10^6 CFU/ml) of an overnight culture of strain WARY9-1M. The cultures were incubated at 37°C for 16-18 h with shaking. Following cultivation, cell-free culture supernatant was obtained through centrifugation at 8,000 x g for 20 min (Sorvall Biofuge, Mandel Scientific, Canada) followed by sterile filtration. Ammonium sulfate (103.2 g) was added to the supernatant while stirring to reach 80% saturation and left overnight at 4°C. The sample was centrifuged at 8000 x g for 50 min. Then the supernatant was discarded and the precipitate was dissolved in 5 ml of sterile distilled water and dialyzed against 1.5 l of sterile distilled water for 16-18 hour. The active supernatant was designated as *partially purified bacteriocin or PPB*.

Spectrum of inhibitory activity

The PPB was used to assess the antibacterial activity against a total of 21 selected Gram-positive and Gram-negative test bacteria by the agar-well diffusion method[9]. Equal volume of sterile distilled water was used as control solution. The appearance of the inhibition zone was determined after 18 h of incubation.

Enzyme sensitivity, heat and pH stability

The PPB was treated at 37°C for 1 h with 1 mg/ml final concentration of the following enzymes: trypsin, α-chymotrypsin and proteinase K (Sigma-Aldrich, USA). After incubation, the reaction mixtures were boiled for 10 min to inactivate the enzymes and the residual antibacterial activity was measured by agar-well diffusion. Thermal stability of bacteriocin was investigated by determination of the residual antibacterial activity after incubation of PPB at different temperature ranging from 40-100°C for 30 and 60 min, and at 121°C for 15 min. To investigate the effect of pH, antibacterial activity was measured following the pH adjustment of the bacteriocin with 0.1 N NaOH or 0.1 N HCl and incubation at 4°C for 1 h.

PCR detection of antimicrobial peptide encoding gene

The genomic DNA of strain WARY9-1M was extracted with E.Z.N.A Bacterial DNA kit (Omega Biotek, USA). Six pairs of primer for amplification of genes involved in synthesis of antimicrobial peptides, lichenysin A, B, C, surfactin, subtilosin and iturin were used in this study (Table 2). PCR amplified products after agarose gel electrophoresis were purified by E.Z.N.A. Gel Extraction Kit (Omega Biotek, USA) and ligated with TA cloning vector (Vivantis, Malaysis). The sequences were determined using M13F forward primer. The nucleotide sequence was compared with GenBank nucleotide database using Blastn search (http://www.ncbi.nlm.nih.gov/blastn).

Molecular weight determination

The molecular weight of bacteriocin from isolated strain WARY9-1M were determined by the Tris-Tricine SDS–PAGE with 5% stacking gel and 16% separating gel[10]. The first half of the gel (protein gel) was stained with PageBlue Protein staining solution (Fermentas, USA) whereas another half of the gel (activity gel) was washed in sterile distilled water for 30 min and overlaid with TSA (0.8% agar) seeded with 1% (v/v) MRSA and incubated at 37°C for 16-18 h. The formation of clear halo or inhibition zone was observed and compared with protein gel.

RESULTS

The isolated strain WARY9-1M was identified as *Bacillus amyloliqufaciens* with 100% identity according to their partial 16S rRNA gene sequence. The antimicrobial activity spectrum of partially purified bacteriocin (PPB) from strain WARY9-1M against 17 test microorganisms was examined by agar diffusion method. The PPB obtained from strain WARY9-1M showed broad antimicrobial activity against both gram-negative and gram-positive test microorganisms. Based on the size of the clear zone, the most sensitive strains were MRSA and *S. aureus*. The complete

inactivation was observed after treatment with all proteolytic enzymes (trypsin, α-chymotrypsin and proteinase K), a key criterion for bacteriocin characterization (Table 1). Temperature stability experiment revealed that PPB was completely stable at high temperature up to 100°C for 60 min (Table 1). With regard to pH sensitivity, antibacterial activity of PPB was maintained at high level within the pH range of 3.0-9.0 (Table 1). PCR was used to analyze for genes involved in the biosynthesis of antimicrobial substances reported to be produced by *Bacillus* sp. PCR analysis of the *B. amyloliquefaciens* strain WARY9-1M showed that the strain exhibited potential for the functional gene encoding malonyl CoA transacylase (*ituD*) and lichenysin synthetase C (*lchAC*) (Figure 1). Sequence of 1,203 bp fragments showed high similarity (99% identity) to malonyl CoA transacylase of *Bacillus amyloliquefaciens* subsp. *plantarum* and only point mutations were observed. The translated nucleotide sequence of *ituD* showed FabD S-malonyltransferase conserved domain at N-terminal part. The molecular weight of bacteriocin from isolated *B. amyloliqufaciens* strain WARY9-1M was determined by Tris-Tricine SDS-PAGE analysis of PPB. As shown in Figure 2, a single protein band with clear halo revealed a bacteriocin activity. The band had an apparent molecular mass of 3 kDa.

Table 1. Effect of enzymes, temperature and pH on PPB of strain WARY9-1M

Treatments and conditions	Residual activity (%)
None (control)	100
Enzyme treatment	
Trypsin	0
α-chymotrypsin	20
Proteinase K	20
Temperature	
40° C, 30 min	100
40° C, 60 min	100
60° C, 30 min	100
60° C, 60 min	100
80° C, 30 min	100
80° C, 60 min	100
100° C, 30 min	100
100° C, 60 min	100
121° C, 15 min	0
pH	
3.0	80
4.0	90
5.0	100
6.0	100
7.0	100
8.0	100
9.0	100

Table 2. The PCR primers used in this study

Primer name	Primer sequence (5'-3')	Target gene	Amplicon size (bp)	Ref.
ituDF	ATGAACAATCTTGCCTTTTTA	Iturin A	1,203	15
ituDR	TTATTTTAAAATCCGCAATT			
LicAF	GTGCCTGATGTAACGAATG	Lichenysin synthetase A	735	16
LicAR	CACTTCCTGCCATATACC	*lchAA*		
LicB2F	TGATCAGCCGGCCGTTGTCT	Lichenysin synthetase B	904	16
LicB2R	GGCGAATTGTCCGATCATGTCC	*lchAB*		
LicCF	GCCTATCTGCCGATTGAC	Lichenysin synthetase C	1,195	16
LicCR	TATATGCATCCGGCACCA	*lchAC*		
sfpF	ATGAAGATTTACGGAATTTA	Surfactin synthetase	675	17
sfpR	TTATAAAAGCTCTTCGTACG			
subsinF	CGCGCAAGTAGTCGATTTCTAACA	Subtilosin	734	18
subsinR	CGCGCAAGTAGTCGATTTCTAACA			

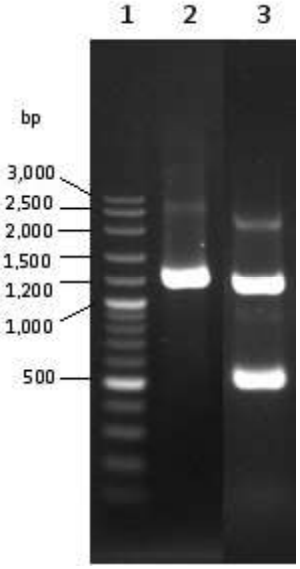

Fig. 1. PCR products profiles of strain WARY9-1M. Lane 1: 100 bp DNA marker, Lane 2 and 3: PCR products obtained from primers specific to iturin A gene and lichenysin synthetase C gene, respectively

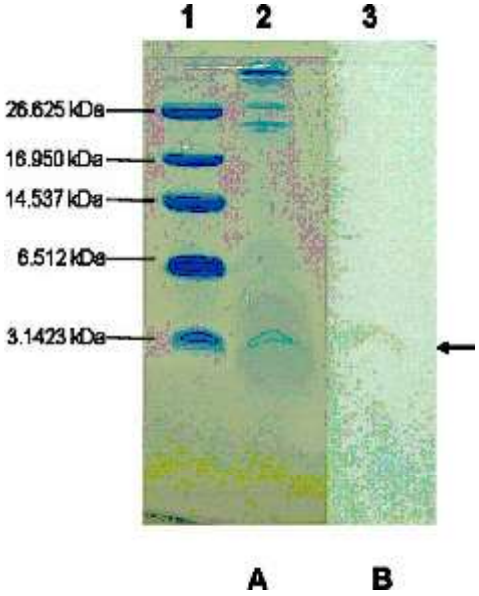

Fig. 2. Tris-Tricine SDS PAGE analysis of PPB prepared from *B. amyloliquefaciens* strain WARY9-1M (A) Coomassie brilliant blue stained gel (B) The activity gel shows the clear zone (arrow) after overlaid with TSA (0.8% agar) seeded with MRSA and incubated overnight. Lane 1: Peptide molecular weight marker; lane 2 and 3: PPB

DISCUSSION

The number of methicillin resistant *Staphylococcus aureus* or MRSA infections has been increasing and become a serious problem in public heath worldwide. Novel antibacterial agents are urgently needed to combat this drug resistant problem. The use of bacteriocin as an alternative agent to overcome the problem is promising[11]. The isolated strain WARY9-1M showing high anti-MRSA was identified as *Bacillus amyloliquefaciens*. It is well documented that bacteriocin or bacteriocin-like production is common among different *Bacillus* species. Most of them can inhibit only gram positive bacteria and less effective against gram negative strains. The partially purified bacteriocin (PPB) prepared from strain WARY9-1M was active against both gram positive and gram negative bacteria under investigation with high antibacterial activity against MRSA and *S. aureus*. The sensitivity of PPB to proteinase K, trypsin and α-chymotrypsin suggests the proteinaceous nature of this antimicrobial substance. Therefore, this solated bacterium can be regarded as bacteriocin-producing bacteria or BAC. The bacteriocin from strain WARY9-1M was heat stable as evidenced by its ability to reserve the activity at 100°C for 60 min. Morevoer, it was stable within a wide range of pH (3-9). The heat stable property was also observed in two bacteriocins, *i.e.*, entomocin 9 and AMS T6-5, produced by *B. thuringienesis* HD9[6] and *B. licheniformis* T6-5 [12], respectively. It was shown by PCR that strain WARY9-1M harbors genes for producing iturin A and lichenysin C. Iturin A and lichenysin C are bacterial cyclic lipopeptides with molecular mass of 1.058 and 1.000 kDa, respectively[13]. The molecular weight of bacteriocin from strain WARY9-1M as determined by Tris-Tricine SDS-PAGE analysis was 3 kDa. The molecular mass of subtilosin reported to be produced by *B. amyloliquefaciens* was 3.399 [14]. However, the strain WARY9-1M did not harbor the gene encoding for subtilosin as analyzed by PCR. This bacteriocin produced by strain WARY9-1M is a novel bacteriocin.

In conclusion, *B. amyloliquefaciens* strain WARY9-1 M produced bacteriocin with high anti-MRSA activity. This strain is non-pathogenic and derived from the nature. The biochemical properties such as thermal stability and wide range pH stability are remarkable. The bacteriocin produced by this microorganism might be used as an alternative source for controlling MRSA in the future.

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