J. Microbiol. Biotechnol. (2009), **19**(11), 1393–1400
doi: 10.4014/jmb.0901.0003
First published online 1 August 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Purification and Characterization of Phocaecin PI80: An Anti-Listerial Bacteriocin Produced by *Streptococcus phocae* PI80 Isolated from the Gut of *Peneaus indicus* (Indian White Shrimp)

Satish Kumar, Ramraj and Venkatesan Arul*

Department of Biotechnology, School of Life Sciences, Pondicherry University, Pondicherry-605014, India

Received: January 3, 2009 / Revised: April 29, 2009 / Accepted: June 15, 2009

A bacteriocin-producing strain PI80 was isolated from the gut of *Penaeus indicus* (Indian white shrimp) and identified as *Streptococcus phocae* PI80. The bacteriocin was purified from a culture supernatant to homogeneity as confirmed by Tricine SDS–PAGE. Reverse-phase HPLC analysis revealed a single active fraction eluted at 12.94 min, and MALDI–TOF mass spectrometry analysis showed the molecular mass to be 9.244 kDa. This molecular mass does not correspond to previously described streptococcal bacteriocins. The purified bacteriocin was named phocaecin PI80 from its producer strain, as this is the first report of bacteriocin production by *Streptococcus phocae*. The bacteriocin exhibited a broad spectrum of activity and inhibited important pathogens: *Listeria monocytogenes*, *Vibrio parahaemolyticus*, and *V. fischeri*. The antibacterial substance was also sensitive to proteolytic enzymes: trypsin, protease, pepsin, and chymotrypsin, yet insensitive to catalase, peroxidase, and diastase, confirming that the inhibition was due to a proteinaceous molecule (*i.e.*, the bacteriocin), and not due to hydrogen peroxide or diacetyl. Phocaecin PI80 moderately tolerated heat treatment (up to 70°C for 10 min) and resisted certain solvents (acetone, ethanol, and butanol). A massive leakage of K^+ ions from *E. coli* DH5α, *L. monocytogenes*, and *V. parahaemolyticus* was induced by phocaecin PI80, as measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Therefore, the results of this study show that phocaecin PI80 may be a useful tool for inhibiting *L. monocytogenes* in seafood products that do not usually undergo adequate heat treatment, whereas the cells of *Streptococcus phocae* PI80 could be used to control vibriosis in shrimp farming.

Keywords: Bacteriocin, *Streptococcus phocae* PI80, *Listeria monocytogenes*, anti-listerial activity, potassium ion (K^+) efflux, probiotics, *Penaeus indicus*, biopreservative

*Corresponding author
Phone: +091-413-2654429; Fax: +091-413-2655265;
E-mail: varul18@yahoo.com

Lactic acid bacteria produce a number of antimicrobial substances, including organic acids, hydrogen peroxide, bacteriocins, and bacteriocin-like substances. Bacteriocins and bacteriocin-like substances are peptides or proteins that exhibit inhibitory activity towards sensitive bacteria strains, and have been grouped into three classes based on their structure [18]: Class I (lantibiotics) are small (<5 kDa) heat-resistant peptides; Class II consists of small (<15 kDa), heat-stable, membrane active, unmodified peptides [28]; and Class III consists of heat-labile proteins with sizes in excess of 15 kDa. The bacteriocins produced from lactic acid bacteria (LAB) are of particular interest owing to their potential use in the food industry as natural, safe food preservatives [5, 26]. This natural approach to food preservation has gained increasing attention and holds promise in view of the increasing concern over the use of chemical preservatives in ready-to-eat products, and in the case of foods that receive minimal thermal treatment during production. In the latter case, such products can develop dangerous levels of pathogenic bacteria, such as *Listeria monocytogenes*, which has been shown to cause serious or even fatal illness and produce numerous outbreaks worldwide [8]. The ability of this pathogen to survive for long periods at refrigerated temperatures [10] and at sodium chloride concentrations of up to 10% [7] makes it a serious health threat, particularly in light of preserved seafood.

Studies of the bacteriocins of streptococci extend back to the 1960s [23]. However, the most recent isolation and characterization of streptococcal bacteriocins have been focused on pathogenic streptococci, where most of the characterized bacteriocins have been found to originate from a few species. Lantibiotics are the most prevalent peptide bacteriocins in streptococci, where most are elongated cationic type A lantibiotics. Two-peptide lantibiotics have also been isolated from streptococci [15].

Streptococcus phocae was first isolated from clinical specimens taken from seals [33], and although there are a few reports on its isolation from seals and Atlantic salmon

[9, 13, 41], there have been no reports of any isolates from shrimp. Moreover, all of the reported isolates have been from diseased animals and were beta hemolytic. However, there has been no previous report on bacteriocin production by *S. phocae*.

Accordingly, the main focus of this study was to characterize an antibacterial substance produced by a non-hemolytic strain, *Streptococcus phocae* PI80, isolated from the gut of Indian white shrimp (*Penaeus indicus*) and then to purify it to homogeneity. This study also attempted to understand the mechanism of the cell damage induced by this antibacterial agent.

Materials and Methods

Strains and Culture Conditions

A bacteriocin producer strain, *Streptococcus phocae* PI80, was previously isolated from the gut of Indian white shrimp (*Peneaus indicus*) in the Senior Author's laboratory [12]. The strain was identified by morphological, physiological, and biochemical tests and a homology search based on the 16S rDNA sequence.

The bacterial strains and media used in this study are listed in Table 1. *E. coli* DH5α was used as the indicator strain for the main test. All the strains were maintained as frozen stocks at −80°C, whereas the working cultures were maintained in an agar medium and subcultured in liquid media before use. An MRS medium (Himedia, Mumbai, India) supplemented with 1% NaCl was routinely used for culturing *Streptococcus phocae* PI80.

Bacteriocin Assay

Liquid cultures inoculated with 0.1% of an inoculum of *Streptococcus phocae* PI80 were grown for 16 h at 37°C with constant shaking at 100 rpm. The cells were then separated by centrifuging the culture medium at 8,500 ×*g* and 4°C for 15 min, whereas the cell-free culture supernatants (CFCS) were neutralized with 1 N sodium hydroxide to pH 6.6±0.1 and used immediately.

A well-diffusion assay procedure was used for the antibacterial assay, as described previously [30]. Eighty μl of the CFCS was placed in a 8-mm well of a TSA (*Vibrios*), BHI (*L. monocytogenes*), and seawater agar plate (*Aeromonas*) (20 ml) previously swabbed (10^{-2} dilution) with the appropriate indicator strain (Table 1). After 24 h of incubation at a temperature optimal for the indicator strain, clear zones of inhibition appeared when the strain was sensitive.

Effects of Heat, Solvent, and Enzyme Treatment on Phocaecin PI80

Dialyzed samples of phocaecin were used in these tests, and *E. coli* DH5α was used as the indicator organism. About 1-ml aliquots of the bacteriocin sample were exposed to temperatures of 50°, 60°, 70°, 80°, 90°, and 100°C for 10, 15, and 30 min and tested for antibacterial activity.

The sensitivity to solvents was determined by incubating the partially purified bacteriocin sample with acetone, ethanol, isopropanol, methanol, butanol, acetonitrile, and chloroform for 1 h at 37°C and testing for antibacterial activity.

Table 1. Growth medium and incubation temperature of indicator strains and inhibitory spectrum of cell-free supernatant of *Streptococcus phocae* PI80.

Indicator strains	Medium and temperature (°C)	Source	Antagonistic activity
Vibrio parahaemolyticus	TSA 37	CIBA, Chennai	++
V. harveyi	Seawater agar 37	Hatchery water	++
V. vulnificus 1145	TSA 37	MTCC, Chandigarh	++
V. fischeri 1738	TSA 37	MTCC, Chandigarh	++
V. anguillarum	TSA 37	CIBA, Chennai	++
Aeromonas. hydrophila	TSA 37	Diseased fish	++
A. hydrophila 646	TSA 37	MTCC, Chandigarh	++
A. salmonicida 1945	TSA 37	MTCC, Chandigarh	++
Escherichia coli DH5α	BHI 37	Reference strain	++
E. coli KL-16	BHI 37	Reference strain	++
E. coli KL-96	BHI 37	Reference strain	++
E. coli CSH-57	BHI 37	Reference strain	-
E. coli SK-39	BHI 37	Reference strain	-
Pseudomonas aeroginosa	BHI 37	Spoilage food	++
Klebsiella pneumonia 30	BHI 37	Human middle ear	++
Proteus vulgaris	BHI 37	CIBA, Chennai	++
Bacillus cereus	BHI 37	Soil	++
Listeria monocytogenes	BHI 37	MTCC, Chandigarh	++
Lactobacillus plantarum	BHI 37	MTCC, Chandigarh	++
L. acidophilus	BHI 37	MTCC, Chandigarh	++
L. rhamnosus	BHI 37	MTCC, Chandigarh	++

+, Inhibition zone less than 10 mm in diameter; ++, inhibition zone larger than 10 mm in diameter; −, no inhibition zone recorded.

The sensitivity to enzymes was determined as described previously [39]. The bacteriocin sample was incubated in the presence of the enzymes protease, chymotrypsin, pepsin, trypsin, lipase, catalase, peroxidase, and diastase, for 2 h at 37°C at a final concentration of 1 mg/ml. All the enzymes were purchased from HiMedia, Mumbai, India. After the incubation, the enzymes were heat inactivated (70°C at 10 min) and tested for inhibition of the indicator bacterium *E. coli* DH5α.

Purification of Phocaecin PI80

The CFCS was obtained as described in "Bacteriocin assays." The supernatant was subjected to Ultrafiltration (GE Healthcare, Uppsala, Sweden) using a 10 kDa cutoff membrane cartridge filter, as the bacteriocin was suspected to be below this molecular mark. Ammonium sulfate was slowly added to the resulting filtrate to produce a 90% saturation (61.3 g/100 ml) and stirred overnight at 4°C. The precipitated proteins were then collected by centrifugation at 10,000 ×*g* for 20 min at 4°C and resuspended in a minimal quantity of a 10 mM ammonium acetate buffer (pH 6.0).The suspension was then dialyzed overnight at 4°C against the same buffer in dialysis tubing (Spectrumlabs, U.S.A.) with a 1 kDa cutoff. Thereafter, the dialyzed sample was applied to a Sephadex G-25 (Sigma, U.S.A.) gel filtration column (C10/20) connected to an Akta Prime plus protein purification system (GE Healthcare, Uppsala, Sweden). The gel column was equilibrated with a 10 mM ammonium acetate buffer (pH 6.0), and 1 ml of the dialyzed sample was eluted using a 10 mM ammonium acetate buffer (pH 6.0) containing 0.01 M sodium chloride at a flow rate of 0.5 ml/min and fraction size of 1.0 ml. For each fraction, a bacteriocin activity assay and protein profiling by Tricine SDS–PAGE were performed.

The active fraction was concentrated using a lyophilizer (Savant, U.S.A.), and the concentrated sample further purified by reverse-phase liquid chromatography (RP–HPLC) [37] using a Shimadzu Analytical HPLC system (Shimadzu, Japan). Briefly, 25 µl of the concentrated bacteriocin (fraction collected from column purification showing inhibition of pathogens) was injected into an analytical C18 reverse-phase column (Luna 5 µm, 4.6×250 mm; Phenomenex, CA, U.S.A.). The elution was performed at a flow rate of 1 ml/min using a linear gradient from 90% solvent A [0.1% (w/v) trifluoro-acetic acid (TFA) in 5% (v/v) acetonitrile in water] and 10% solvent B (0.1% TFA in 100% acetonitrile) to 42% and 58% of solvents A and B, respectively, within 46 min. The peptide fractions were detected spectrophotometrically by measuring the absorbance at 220 nm and collected manually. The fractions were then concentrated using a lyophilizer and dissolved in an ammonium acetate buffer (10 mM, pH 6.0) for use in the bacteriocin activity assay and molecular mass determination.

Molecular Mass Determination

The molecular size of phocaecin PI80 was determined by separation of the fraction obtained after an HPLC analysis using Tricine SDS–PAGE (10%) [31]. A low molecular mass protein marker with sizes ranging from 3.0 to 205 kDa (Bangalore Genei, Bangalore, India) was used. The gel was stained using the silver staining method [24].

The molecular mass of phocaecin PI80 was further confirmed by mass-assisted laser desorption ionization time of flight (MALDI–TOF) mass spectrometry. The mass of the peptide was determined by the Proteomic facility of the Molecular Biophysics Unit at the Indian Institute of Sciences, Bangalore, India.

Bacteriocin Activity in Polyacrylamide Gels

The Tricine SDS–PAGE was run under nonreducing conditions [35]. The boiling and addition of 2-mercaptoethanol in the probe buffer was suspended. The gel was washed 4 times for 10 min in distilled water with gentle agitation to remove the SDS. The gel was placed in a petri dish containing a 2% agar medium and then overlaid with a precooled 0.7% agar medium inoculated with the indicator organism. The plate was incubated for 24 h at 37°C.

Mode of Action and Determination of K^+ Efflux by Phocaecin PI80

The mode of action of phocaecin PI80 was determined by measuring the potassium ion leakage in the external medium [11]. Briefly, the *Streptococcus phocae* PI80 cells were grown in an MRS broth at 37°C for 16 h. The phocaecin PI80 was then partially purified from the culture filtrate using Sephadex G-25 gel chromatography (Akta prime plus) and the bacteriocin activity found to be 1,280 AU/ml. The indicator strains *E. coli* DH5α, *E. coli* CSH57, *L. monocytogenes*, and *V. parahaemolyticus* were grown overnight at 37°C and diluted to 10^{-2}. Equal volumes of the purified bacteriocin sample and indicator bacteria were incubated for 1 h at 37°C and centrifuged to remove the particulate material. The bacteriocin+water served as the common control for the experiment, whereas a second control of just the bacterial cells was kept as an individual indicator organism so as to rule out any K^+ interference due to the medium or cell lysis caused by reasons other than the bacteriocin. The standards (1–100 ppm) were prepared by dissolving KCl in distilled water. The K^+ efflux was determined by measuring the concentration of K^+ using Inductively Coupled Plasma Optical Emission Spectrometry (ICP–OES) (Jobin Vyon, Japan).

RESULTS

Inhibitory Spectrum of Phocaecin PI80

Inhibition was found towards a wide spectrum of bacterial species, including both Gram positive and negative (Table 1). Phocaecin PI80 was also found to be active against important pathogens in food and livestock (fish and shrimp): *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, and *Pseudomonas aeroginosa.*

Effects of Heat, Solvents, and Enzymes on Phocaecin PI80

Phocaecin PI80 was found to be heat resistant at temperatures of up to 70°C for 10 min. At temperatures of 50°C, 60°C, 70°C, and 80°C for 10 min, 100%, 44%, 22%, and 11% activity was retained, respectively (Table 2). However, at higher temperatures (>80°C) and when the exposure time was extended to 15 min and longer, phocaecin PI80 became unstable. It was sensitive to isopropanol, methanol, acetonitrile, and chloroform, yet resistant to acetone, ethanol, and butanol (Table 2). It was also sensitive to pepsin, papain, chymotrypsin, trypsin, and protease, yet insensitive to catalase, peroxidase, and diastase, confirming that the inhibition was due to a proteinaceous molecule (*i.e.*, bacteriocin) and

Table 2. Effects of heat, solvents, and enzymes on bacteriocin sample from *Streptococcus phocae* PI80.

Treatment	Bacteriocin activity[a]
Heat	
50°C for 10 min.	+
60°C for 10 min.	+
70°C for 10 min.	+
80°C for 10 min.	+
90°C for 10 min.	–
Solvents	
Acetone	+
Ethanol	+
Isopropanol	–
Methanol	–
Butanol	+
Acetonitrile	–
Chloroform	–
Enzymes	
Trypsin	–
Diastase	+
Catalase	+
Protease	–
Lipase	+
Pepsin	–
Chymotrypsin	–
Peroxidase	+

[a]Bacteriocin activity was determined against *E. coli* DH5α.
+, Inhibition zone; –, no inhibition zone.

not due to hydrogen peroxide or diacetyl. Furthermore, its activity was not reduced by lipase, indicating no structural modification due to the lipid moiety (Table 2).

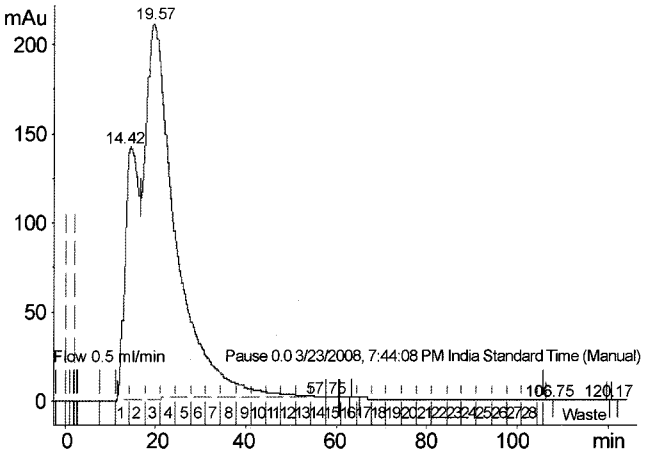

Fig. 1. Sephadex G-25 chromatogram for bacteriocin sample.
The bacteriocin sample was purified by Sephadex G-25 gel chromatography and eluted at a flow rate of 0.5 ml/min. The bacteriocin protein was eluted at about 19.57 min in fraction numbers 4 to 10.

Purification of Phocaecin PI80

To purify phocaecin PI80, the cell-free culture supernatant (CFCS) was ultrafiltered through a 10 kDa membrane, precipitated with ammonium sulfate, dialyzed, and subjected to Sephadex G-25 gel chromatography (Fig. 1), as described in "Materials and Methods." The resulting active fraction was found to retain 44% of the initial activity found in the CFCS. This active fraction was lyophilized and dissolved in a minimum volume of Solvent A for an HPLC analysis. Upon injection of the Sephadex G-25-purified sample into the reverse-phase HPLC, a distinct peak was eluted at 27.34% of acetonitrile, corresponding to a retention time of 12.94 min (Fig. 2). The peak was also shown to be

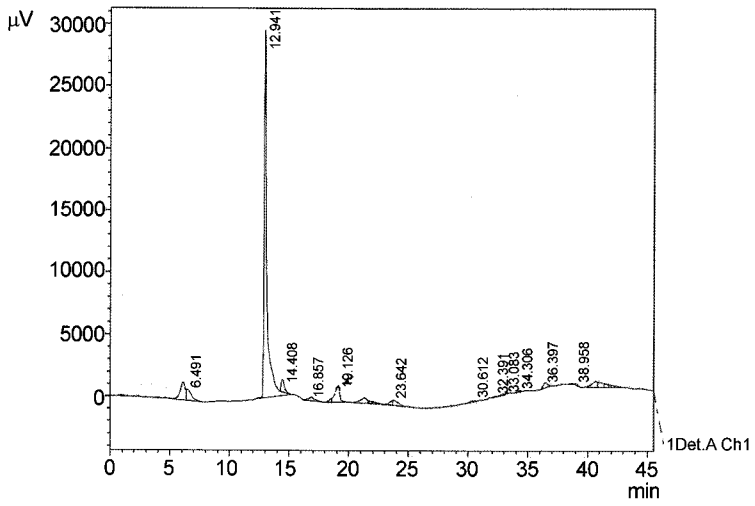

Fig. 2. High-performance liquid chromatography (HPLC) chromatogram.
The lyophilized partially purified samples were resuspended in a minimal quantity of solvent A. A 25-μl sample was eluted using a linear gradient from 90% solvent A [0.1% (w/v) trifluoroacetic acid (TFA) in 5% (v/v) acetonitrile in water] and 10% solvent B (0.1% TFA in 100% acetonitrile) to 42% and 58% of solvents A and B, respectively, within 46 min. A distinct peak was obtained at 12.941 min.

active against *E. coli* DH5α and used for the molecular mass determination.

Molecular Mass Determination

The Tricine SDS–PAGE of the active fraction collected from the RP–HPLC analysis yielded a peptide band of 7.3 kDa (Fig. 3). Furthermore, the MALDI–TOF mass spectra of the HPLC-purified protein revealed a sharp peak corresponding to 9.24 kDa (Fig. 4), indicating a molecular mass of 9.24 kDa.

Bacteriocin Activity in Polyacrylamide Gels

Clear areas appeared around the zones of migration of phocaecin PI80 when the gel was incubated with *E. coli* DH5α (Fig. 3), corresponding to the bacteriocin band obtained after purification.

Mode of Action of Phocaecin PI80

The potassium ion leakage by the susceptible cells due to the action of phocaecin PI80 alone was assessed by subtracting the value obtained (ppm) from the test reaction (bacteriocin+cells) from the control values for the bacteriocin (control a) and bacterial cells (control b). After incubating *E. coli* DH5α, *L. monocytogenes*, and *V. parahaemolyticus* with the partially purified bacteriocin sample, a K^+ efflux of 983.3±17.5 ppm, 603.3±13.0 ppm, and 556.6±14.6 ppm was observed, respectively, in ICP–OES. When subtracting these values from the control K^+ ion efflux due to phocaecin PI80 alone, this resulted in 434 ppm, 98 ppm, and 118.7 ppm for *E. coli* DH5α, *L. monocytogenes*, and *V. parahaemolyticus*, respectively. Thus, when compared with the control, these values were significant. However, *E. coli* CSH57, which served as the negative control, did not show any significant level of K^+ ions in the sample (Table 3).

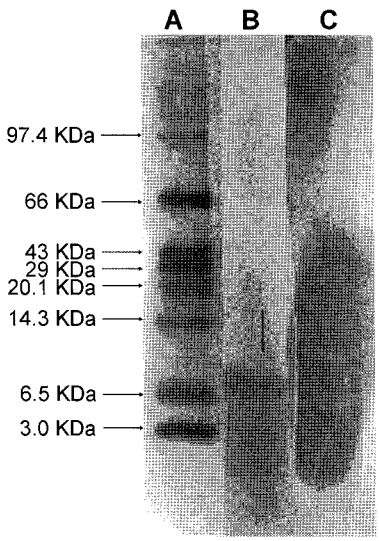

Fig. 3. Tricine SDS–PAGE of purified bacteriocin from *Streptococcus phocae* PI80.

A. Molecular mass marker (3.0–205 kDa). **B**. Purified active bacteriocin sample showing a single band of 7.3 kDa. **C**. Nonreducing Tristricine SDS–PAGE in a 10% migrating gel overlaid with agar (0.7%) inoculated with the sensitive strain *E. coli* DH5α. An activity zone corresponding to a band of approximately 7.3 kDa provided evidence of antibacterial activity.

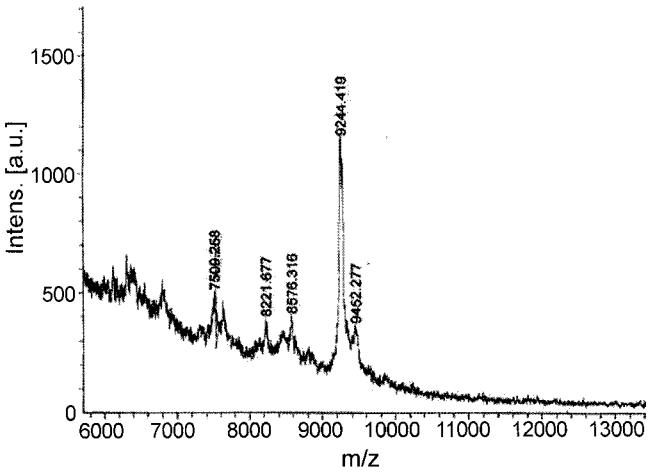

Fig. 4. Mass-assisted laser desorption ionization time of flight (MALDI–TOF) mass spectrum of purified bacteriocin.

The single active peak fraction of HPLC was subjected to mass spectrometry. The MALDI–TOF analysis showed a distinct peak at 9244.419 *m/z*, indicating that the molecular mass of the bacteriocin protein was 9.244 kDa.

Discussion

Phocaecin PI80 is a novel bacteriocin isolated from *Streptococcus phocae* PI80 with a broad spectrum of antimicrobial activity, including both Gram-positive and Gram-negative bacteria. *Streptococcus phocae* was first isolated from seals [33] and then from Atlantic salmon [9]. Previous reports on *S. phocae* have identified it as beta hemolytic and mostly isolated from diseased animals [9, 34]. However, the *S. phocae* was isolated from shrimp in the Senior author's laboratory, and the strain was found to be nonhemolytic and nonpathogenic, which was confirmed by testing the strain with the common carp *Cyprinus carpio* [12] and shrimp *Penaeus monodon* [29]. Moreover, no previous report has been found regarding the isolation of bacteria from *Penaeus indicus* for the purpose of bacteriocin production and probiotics. Therefore, this is the first report of a bacteriocin from *S. phocae*. Phocaecin PI80 exhibited strong anti-listeric activity against *L. monocytogenes*. Other Streptococcal strains that also show anti-listerial activity include *S. thermophilus* 110 [34], *S. thermophilus* 13 [20], *S. thermophilus* 81 [16], and *S. thermophilus* 347 [40]. Bacteriocins that are active against *L. monocytogenes* have already been used successfully for food preservation. For example, nisin at a concentration of 2.5 mg/l effectively inhibits the growth of *L. monocytogenes* in ricotta-type

Table 3. ICP-OES determination of K^+ concentration (ppm) induced by phocaecin PI80.

Samples	K^+ concentration in cell-free supernatant (ppm)	K^+ efflux due to phocaecin PI80 (ppm)
Water+phocaecin PI80[a]	301.3±11.0	
L. monocytogenes[b]	204.0±11.0	
L. monocytogenes+phocaecin PI80[c]	603.3±13.0*	+98.00
V. parahaemolyticus[b]	136.6±8.5	
V. parahaemolyticus+phocaecin PI80[c]	556.6±14.6*	+118.70
E. coli DH5α[b]	247.6±13	
E. coli DH5α+phocaecin PI80[c]	983.3±17.5*	+434.40
E. coli CSH57[b]	238.6±10.0	
E. coli CSH57+phocaecin PI80[c]	272.60±13.0	-267.30

[a]Common control for whole experiment.
[b]Control for individual bacterium.
[c]Test reaction.
*Significant at $p<0.05$ level.
K^+ leakage induced by effect of phocaecin PI80 alone=c-(a+b).

cheese for a period of 8 weeks or more [6]. In addition, bacteriocins have also been found to be effective against *L. monocytogenes* when used in combination with an increased NaCl concentration [38]. As *S. phocae* has already shown antagonistic activity towards *Aeromonas hydrophila*, *V. parahaemolyticus*, *V. harveyi*, *V. fischeri* 1738, and *V. anguillarum*, it is suggested that *Streptococcus phocae* PI80 may be effective as a probiotic agent for shrimp farming, as well as in the preservation of seafood to enhance the shelf life.

In this study, phocaecin PI80 was found to be heat stable up to 70°C for 10 min, making it neither very heat stable nor very heat labile, whereas bacteriocins previously extracted from *Streptococcus* species are either heat stable or heat labile [16, 21, 34, 36]. Phocaecin PI80 also showed sensitivity towards proteolytic enzymes, protease, chymotrypsin, pepsin, and trypsin, suggesting a proteinaceous nature. Similar characteristics have been observed for *Thermophilin* 110 [34], *Thermophilin* 580 [21], and *Thermophilin* 13 [20].

Phocaecin PI80 was purified to homogeneity, and MALDI-TOF mass spectrometry confirmed the purity of the sample and determined the molecular mass of the bacteriocin to be 9.24 kDa. In the case of SDS-PAGE, the bacteriocin migrated with an apparent molecular mass of about 7.3 kDa. This discrepancy could be attributed to the nonlinear migration of small peptides on SDS-PAGE gels [27]. Similarly, lacticin H-559 and gassericin A migrate to positions of 2.5 kDa and 3.8 kDa respectively, in the case of SDS-PAGE, whereas mass spectrometry shows their molecular mass to be 3.34 kDa and 5.65 kDa, respectively [14, 17]. Phocaecin PI80 could be classified as a class II bacteriocin based on its molecular mass [18]. Some of the bacteriocins that have already been isolated from bacteria belonging to the genus *Streptococcus* have either a low molecular mass or high molecular mass [15]. Dysgalactin isolated from *Streptococcus dysgalactiae* subsp. *equisimilis* has a molecular mass of 21 kDa. [42] Similarly, Stellalysin (29 kDa) and SA-M57 (17 kDa) were isolated from *Streptococcus constellatus* subsp. *constellatus* and M-type 57 *Streptococcus pyogenes*, respectively [32, 42].

In most previous studies, the mode of bacteriocin activity is typically monitored by a decrease in the viability of an indicator organism [2, 34], yet this effect alone provides little information on the bacteriocin action. Previous studies have indicated that LAB bacteriocins are pore-forming peptides that catalyze the potassium efflux from sensitive strains [1, 19, 22]. Similarly, phocaecin PI80 induced a massive leakage of K^+ from *L. monocytogenes*, *E. coli* DH5α, and *V. parahaemolyticus*, when increasing the outside concentration. Therefore, this finding suggests that phocaecin PI80 renders the membrane of sensitive cells permeable, allowing the efflux of K^+. This release also probably occurs through pore formation [4, 25], where the resultant pore formation in the cytoplasmic membrane induces the leakage of small intracellular substances from sensitive cells [3]

Consequently, to establish a safe and effective antimicrobial agent, additional investigation is currently under way to further characterize the efficacy of phocaecin PI80 as a seafood biopreservative and *S. phocae* PI80 as a potential probiont for shrimp farming.

Acknowledgments

This work was supported by a grant from the Department of Biotechnology (DBT), India. R.S.K. was supported by an ICMR fellowship. The authors would also like to thank Dr. Pramod Singh, Department of Earth Sciences, Pondicherry University for the use of the ICP-OES facility.

REFERENCES

1. Abee, T., T. R. Klaenhammer, and L. Letellier. 1994. Kinetic studies of the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii* that forms poration complexes in the cytoplasmic membrane. *Appl. Environ. Microbiol.* **60:** 1006–1013.
2. Amer, A. E. 2007. Characterization of a bacteriocin-like inhibitory substance produced by *Lactobacillus plantarum* isolated from Egyptian home made yoghurt. *Science Asia* **33:** 313–319.
3. Beatriz, G., G. Erwin, R. S. Edmund, J. M. Arnold, E. S. Juan, and N. K. Wil. 1996. Bactericidal mode of action of plantaricin C. *Appl. Environ. Microbiol.* **62:** 2701–2709.
4. Castellano, P., R. Raya, and G. Vignolo. 2003. Mode of action of lactocin 705, a two-component bacteriocin from *Lactobacillus casei* CRL705. *Int. J. Food Microbiol.* **85:** 35–43.
5. Cleveland, J., T. J. Montville, I. F. Nes, and M. L. Chikindas. 2001. Bacteriocins: Safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.* **71:** 1–20.
6. Davies, E. A., H. E. Bevis, and B. J. Delves. 1997. The use of the bacteriocin nisin, as a preservative in ricotta-type cheese to control the food-borne *Listeria monocytogenes*. *Lett. Appl. Microbiol.* **24:** 343–346.
7. Farber, J. M. and P. I. Peterkin, 1991. *Listeria monocytogenes*, a foodborne pathogen. *Microbiol. Rev.* **55:** 476–511.
8. Gahan, C. G. and J. K. Collins. 1991. Listeriosis: Biology and implication for the food industry. *Trends Food Sci. Tech.* **2:** 89–93.
9. Gibello, A., A. I. Mata, M. M. Blanco, A. Casamayor, L. Dominguez, and F. Jose Fernandez-Garaysabal. 2005. First identification of *Streptococcus phocae* isolated from Atlantic salmon (Salmo-Salar). *J. Clin. Microbiol.* **43:** 526–527.
10. Gill, C. O. and M. P. Reichel. 1989. Growth of the cold-tolerant pathogens *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* on high-pH beef packaged under vacuum or carbon dioxide. *J. Food Microbiol.* **6:** 223–230.
11. Ghrairi, T., M. Frère, J. M. Berjeaud, and M. Manai. 2005. Lactococcin MMT24, a novel two-peptide bacteriocin produced by *Lactococcus lactis* isolated from rigouta cheese. *Int. J. Food Microbiol.* **105:** 389–398.
12. Gopalakannan, A. 2006. Studies on the control of *Aeromonas hydrophila* infection in *Cyprinus carpio* and *Tilapia mossambicus* by immunostimulants and probiotics. PhD Thesis. Pondicherry University, Pondicherry, India
13. Henton, M. M., O. Zapke, and P. A. Basson. 1999. *Streptococcus phocae* infections associated with starvation in cape fur seals. *J. S. Afr. Vet. Assoc.* **70:** 98–99.
14. Hun, J. L., J. J. Yun, S. P. Chan, H. K. Seung, I. H. Kyeong, S. A. Jong, and I. M. Tae. 1999. Purification and characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* H-559 isolated from kimchi. *J. Biosci. Bioeng.* **88:** 153–159.
15. Ingolf, F. S., B. D. Dzung, and H. Holo. 2007. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. *J. Bacteriol.* **189:** 1189–1198.
16. Ivanova, I., V. Miteva, T. S. Stefanova, A. Pantev, I. Budakov, S. Danova, *et al.* 1998. Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. *Int. J. Food Microbiol.* **42:** 147–158.
17. Kawai, Y., T. Saito, M. Suzuki, and T. Itoh. 1998. Sequence analysis by cloning of the structural gene of gassericin A, a hydrophobic bacteriocin produced by *Lactobacillus gasseri* LA 39. *Biosci. Biotechnol. Biochem.* **62:** 887–892.
18. Klaenhammer, T. R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* **12:** 39–85.
19. Mantovani, H. C., H. Hu, R. W. Worobo, and J. B. Russell. 2002. Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. *Microbiology* **148:** 3347–3352.
20. Marciset, O., J. M. C. Stratingh, B. Mollet, and B. Poolman. 1997. Thermophilin 13, a nontypical antilisterial poration complex bacteriocin, that functions without a receptor. *J. Biol. Chem.* **272:** 14277–14284.
21. Mathot, A. G., E. Beliard, and D. Thuault. 2003. *Streptococcus thermophilus* 580 produces a bacteriocin potentially suitable for inhibition of *Clostridium tyrobutyricum* in hard cheese. *J. Dairy Sci.* **86:** 3068–3074.
22. McAuliffe, O., M. P. Ryan, R. P. Ross, C. Hill, P. Breeuwer, and T. Abee. 1998. Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. *Appl. Environ. Microbiol.* **64:** 439–445.
23. Mindich, L. 1966. Bacteriocins of *Diplococcus pneumoniae*. I. Antagonistic relationships and genetic transformations. *J. Bacteriol.* **92:** 1090–1098.
24. Morrissey, J. H. 1981. Silver staining for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* **117:** 307–310.
25. Nes, I. F. and H. Holo. 2000. Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers* **55:** 50–61.
26. O'Sullivan, L., R. P. Ross, and C. Hill. 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie* **84:** 593–604.
27. Piard, J. C., P. M. Muriana, M. J. Desmazeaud, and T. R. Klaenhammer. 1992. Purification and partial characterization of lacticin 481, a lanthionine-containing bacteriocin produced by *Lactobacillus lactis* subsp. *lactis* CNRZ 481. *Appl. Environ. Microbiol.* **58:** 279–284.
28. Sablon, E., B. Contreras, and E. J. Vandamme. 2000. Antimicrobial peptides of lactic acid bacteria: Mode of action, genetics and biosynthesis. *Adv. Biochem. Eng. Biotechnol.* **68:** 21–60.
29. Sandip, M. S., C. Singh, and V. Arul. 2009. Inhibitory activity of *Streptococcus phocae* PI80 and *Enterococcus faecium* MC13 against vibriosis in shrimp *Penaeus monodon*. *World J. Microbiol. Biotech.* **25:** 697–703.
30. Schillinger, V. and F. K. Lucke. 1989. Antimicrobial activity of *Lactobacillus sake* isolated from meat. *Appl. Environ. Microbiol.* **39:** 189–195.
31. Schägger, H. and V. G. Jagow. 1987. Tricine-sodium dodecyl sulphate polyacrilamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166:** 368–379.
32. Simpson, W. J. and J. R. Tagg. 1983. M-Type 57 group A *Streptococcus* bacteriocin. *Can. J. Microbiol.* **29:** 1445–1451.
33. Skaar, I., P. Gaustad, T. Tonjum, B. Holm, and H. Stenwig. 1994. *Streptococcus phocae* sp. nov., a new species isolated from clinical specimens from seals. *Int. J. Syst. Bacteriol.* **44:** 646–650.
34. Stefanie, E. G. and A. S. George. 2005. Thermophilin 110: A bacteriocin of *Streptococcus thermophilus* ST110. *Curr. Microbiol.* **51:** 175–182.
35. Svetoslav, G. D. 2007. A novel bacteriocin-like substance produced by *Enterococcus faecium* 3587. *Curr. Microbiol.* **55:** 323–327.

36. Tadashi, I., I. Takefumi, M. Hirasawa, C. Watanbe, R. M. Jerry, and T. Shiota. 1982. Purification and certain properties of bacteriocin from *Streptococcus mutans*. *Infect. Immun.* **35:** 861–868.
37. Tahiri, I., M. Desbiens, R. Benech, E. Kheadr, C. Lacroix, S. Thibault, D. Ouellette, and I. Fliss. 2004. Purification, characterization and amino acid sequencing of divergicin M35: A novel class IIa bacteriocin produced by *Carnobacterium divergens* M35. *Int. J. Food Microbiol.* **97:** 123–136.
38. Thomas, L. V. and J. W. Wimpenny. 1996. Investigation of the effect of combined variations in temperature, pH, and NaCl concentration on nisin inhibition of *Listeria monocytogenes* and *Staphylococcus aureus*. *Appl. Environ. Microbiob.* **62:** 2006–2012.
39. Tiwari, S. K. and S. Srivastava. 2008. Purification and characterization of plantaricin LR14: A novel bacteriocin produced by *Lactobacillus plantarum* LR/14. *Appl. Microbiol. Biotechnol.* **79:** 759–767.
40. Villani, F., O. Pepe, G. Mauriello, G. Salzano, G. Moschetti, and S. Coppola. 1995. Antilisterial activity of thermophilin 347, a bacteriocin produced by *Streptococcus thermophilus*. *Int. J. Food Microbiol.* **25:** 179–190.
41. Vossen, A., A. Abdulmawjood, C. Lämmler, R. Wein, and U. Siebert. 2004. Identification and molecular characterization of beta-hemolytic streptococci isolated from harbor seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) of the German North and Baltic Seas. *J. Clin. Microbiol.* **42:** 469–473.
42. Wong, H. K. 1981. Bacteriocin-like inhibitors of group A streptococci produced by group F and group G streptococci. *Proc. Univ. Otago Med. Sch.* **59:** 105–106.