Original Article

Screening for Bacteriocin-Like Antimicrobial Activity Against Shrimp Pathogenic Vibrios and Molecular Identification of Marine Bacteria from Otter Clam *Lutraria philippinarum*

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Abstract

This study aimed to isolate marine bacteria from otter clam *Lutraria philippinarum*, screen for bacteriocin-like antimicrobial activity against shrimp pathogenic vibrios and identify these bacteria by molecular methods. Among a total of 128 bacterial isolates, 19 (15%) were found to produce bacteriocins against at least one of three shrimp pathogenic *Vibrio* strains. The highest bacteriocin production activity was shown by six strains, named H9, H18, H51, H61, H77 and H108. The antimicrobial activity of crude bacteriocin extracts from these strains was completely inactivated after proteinase K and trypsin treatment, heat-labile but relatively stable at pH 4-10. Sequencing and phylogenetic analysis of the 16S rRNA and *rpoB* genes resulted in the identification of one strain (H77) as *Enterobacter cloacae* and the other strains as *Cronobacter sakazakii*. This is the first evidence of bacteriocins produced by a member of the genus *Cronobacter* and also by bacteria associated with clam. The research presents data that support further studies of bacteriocin diversity in marine bacteria and their potential as probiotics or antibiotic substitutes for sustainable aquaculture.

Keywords: antimicrobials, bacteriocins, *Cronobacter*, probiotics, shrimp, *Vibrio*

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Introduction

Shrimp cultivation is one of the most economically important aquacultural activities in Asia and worldwide. However, farmers have faced serious diseases of opportunistic bacterial pathogens, in which *Vibrio* species is the most important pathogens. Among them, luminous *V. harveyi* strains, which sometimes caused a 100% loss in shrimp production (Flegel, 2012; Zokaeifarl et al., 2012), have been most well studied. In 2009, a new, emerging disease called "early mortality syndrome" (EMS) or "acute hepatopancreatic necrosis syndrome" (AHPNS) began to cause heavy losses in penaeid shrimp production in southern China, and then expand through whole China, Vietnam, Malaysia and Thailand during 2010-2013, greatly impacting on global seafood markets. The pathogenic agent of AHPNS has recently been determined as *V. parahaemolyticus* and other members of *V. harveyi* clade (Tran et al., 2013).

In addition, due to overuse in aquaculture, classical antibiotics present of disease resistance by bacteria, damaged normal microflora and caused microdysbiosis. It also made residues accumulated in aquatic products to be harmful for human (Gillor et al., 2008). Thus, scientific communities have proposed friendly alternatives such as the use of vaccines, antibiotic substitutes and probiotics (Nguyen et al., 2013). However, vaccine use is often laborious, costly and stressful to the animals. Therefore, bacteriocinogenic bacteria appear to be an excellent candidate with dual role because bacteriocin would be an antibiotic substitute, whereas bacteria would be potential probiotics (Gillor et al., 2008).

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins produced by bacteria to inhibit the growth of similar or closely related bacterial strains (Riley, 2009). Two main features distinguish many bacteriocins from classical antibiotics: bacteriocins are ribosomally synthesized (Desriac et al., 2010) and often have a relatively narrow killing spectrum (Cotter et al., 2005). For example, bacteriocins from lactic acid bacteria are directed against low-GC Gram-positives in general and sometimes against Gram-negatives when the integrity of their outer membrane has been compromised (Riley, 2009).

Bacteriocins are classified mainly based on producer bacterial family, their molecular weight, their amino acid sequence homologies and/or gene cluster organization. Although the systems for bacteriocin naming are still problematic, bacteriocins are usually divided into four classes. Class I, antibiotics, is composed of post-translationally modified bacteriocins. Class II includes heat-stable, minimally modified bacteriocins. Class III includes larger, heat-labile bacteriocins. Class IV comprises complex bacteriocins carrying lipid or carbohydrate moieties. Bacteria strains which produce bacteriocins classes I and II have been suggested as promising probiotics (Riley, 2009).

Bacteriocins have been studied for positive health benefits to the host including human, livestock, aquaculture animals and some plants (Riley and Wertz, 2002). For using bacteriocin as potential probiotics and antibiotic substitutes in aquaculture, bacteriocinogenic bacteria must be isolated from marine animals and marine-environment to be able to adapt to the change of temperature and salinity in farming conditions (Desriac et al., 2010). Thus, local marine animals might be suitable for being isolated and screened for new potential probiotics and antibiotic substitutes. In a parallel study, we just reported the first evidence of bacteriocin production by marine bacteria associated with lobster, tiger shrimp, snubnose pompano and cobia, in which two novel bacteriocinogenic probiotics provided additional options for sustainable management of vibriosis in ornate spiny lobster (*P. ornatus)* (Nguyen et al., 2014). In the present research, otter clam (*Lutraria philippinarum)* was chosen to screen potential probiotics as a part of a strategy to develop a sustainable aquaculture industry for shrimp. In our knowledge, it is the first time that a marine bacterium with bacteriocin-like antimicrobial activity was found in clam and also the first bacteriocins produced by a member of the *Cronobacter* genus.

Materials and Methods

Bacterial isolation from otter clam: Otter clams (*Lutraria philippinarum*) (n=9) were collected from Nha Trang Bay and Cam Ranh Bay, Khanh Hoa province, Vietnam, from February to October 2012. The intestinal content and mucus of clams were prepared and then plated on Trypticase Soy Agar (Difco, Detroit, MI, USA) supplemented with 1.5% NaCl for bacteria isolation as described by Balcázar et al. (2010).

Assay for bacteriocin-like antimicrobial activity: Antimicrobial activity was determined by the agar-well diffusion method as described by Zokaeifa et al. (2012) with modifications as follow. Isolates were grown in Trypticase Soy Broth (Difco) for harvesting cell-free supernatants of which pH was then adjusted to 7.0 to remove the effect of organic acid and which were treated with catalase (Promega, Madison, WI, USA) to remove the effect of hydrogen peroxide. Plates were prepared with TCBS agar (Difco) containing 10^6 cells per ml of shrimp pathogens as indicator bacteria including *Vibrio parahaemolyticus* strains C1 and V1.1, and *V. alginolyticus* V3.3, which were obtained from Nha Trang University Culture Collection (NTUCC). These strains were isolated from black tiger shrimp (*Penaeus monodon*) that died with typical gross signs of AHPNS (Tran et al., 2013). To check the proteinaceous nature of the inhibiting compound, the supernatant fluids were treated with trypsin (Promega) according to manufacturer's instructions.

Effect of physiochemical factors on the activity of bacteriocin extracts: The supernatant fluids' pH was first adjusted to 7 and they were treated with catalase as described above. Then, they were treated with different enzymes (proteinase K, trypsin, lipase, α-amylase (Promega)) as recommended by the manufacturer, temperatures (30°C, 60°C, or 100°C for 30 min, 121°C for 15 min) and pH levels (2-12) for 30

min. Residual activity after the treatment was determined using the agar-well diffusion assay.

Genomic DNA extraction and PCRs of 16S rRNA and rpoB genes: DNA of bacterial strains was extracted by alkaline lysis method using kit Wizard®SV Genomic DNA Purification System (Promega). Purified DNA samples were used as templates for amplification of 16S rRNA and *rpoB* gene segments using respective primers (Integrated DNA Technologies, Coralville, IA, USA) as follows: 16S-27F and 16S-1492R (Luan et al., 2007), CM7 and CM31b (Mollet et al., 1997). PCRs were performed as described by Luan et al. (2007).

Gene sequencing and phylogenetic analyses: The PCR product of the 16S rRNA and *rpoB* genes of bacterial isolates was purified using PCR Clean Up System Kit (Promega) and used as template for sequencing using Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130XL DNA Sequencer (Applied Biosystems) in Nam Khoa Biotek Company, Hochiminh city, Vietnam and an ABI 3730XL DNA Sequencer (Applied Biosystems) in Marcrogen Company, Seoul, Korea. The 16S rRNA and *rpoB* gene sequences of bacterial isolates and reference sequences available in GenBank were used for sequence analysis at the National Center for Biotechnology Information (NCBI) using BLAST. Phylogenetic trees were constructed using MEGA program ver. 5.2.1 (Tamura et al., 2011). Putative bacteriocins were identified by mining the known complete genome of *Cronobacter* and *Enterobacter* strains using BAGEL (http://bagel.molgenrug.nl) (De Jong et al., 2010).

Production of bacteriocins and their effect on indicator bacterium: To define the maximum bacteriocin production activity, the culture extracts of the bacteriocin-producing strain (*C. sakazakii* H108) in Trypticase Soy Broth (Difco) at 37°C were collected every 3 h during the growth. The growth was determined by optical density values at 600 nm (OD_{600}). Crude bacteriocin extracts were harvested by centrifugation at 8000 × g for 10 min at 4°C to remove cell pellets. Bacteriocin activity was determined by the agar-well diffusion method as described above and was expressed as arbitrary units (AU) per mL. One AU was defined as reciprocal of the highest dilution showing a clear zone of growth inhibition (Todorov and Dicks, 2009).

To determine the effect of bacteriocins on growing cells of a sensitive bacterial strain (infection types: bactericidal, bacteriostatic, bacteriolysis), the indicator bacterium (*V. parahaemolyticus* V1.1) was grown in Trypticase Soy Broth (Difco) at 37°C. Crude bacteriocin extracts of H108 at different concentrations were added to early exponential phase cultures (≈10^6 CFU/mL) of the indicator. The growth of the indicator was determinded by optical density values at 600 nm (OD_{600}) and measured every hour during 10 h. Cultures without added bacteriocins were used as controls. In addition, the determination of cell lysis and the reduction of viable cells of indicator bacteria in the presence of bacteriocins was also carried out as described by Todorov and Dicks (2009).

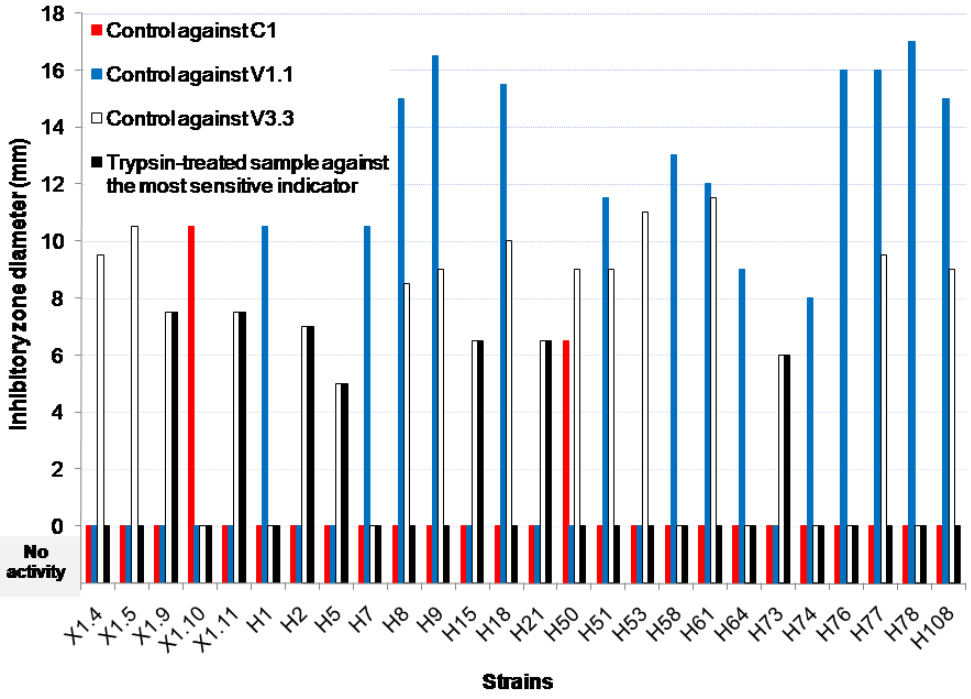

Figure 1 Antimicrobial activity of isolates from otter clam against three shrimp pathogenic vibrios, C1, V1.1 and V3.3, compared with trypsin-treated samples against the most sensitive indicator. The values zero of inhibitory zone diameter express no activity of samples. All presented zone diameters are an average of six results.

Results

Isolation and screening of marine bacteria with bacteriocin-like antimicrobial activity from otter clam: A total of 128 strains were isolated from otter clam (*L. philippinarum*). Of these, 26 strains exhibited inhibitory activity against at least one of three shrimp pathogens as indicator bacteria (Fig 1). In particular, 14 strains could inhibit the growth of *V. parahaemolyticus* V1.1, 19 strains against *V. alginolyticus* V3.3, and two strains against *V. parahaemolyticus* C1. Among them, eight strains showed inhibitory activity against both V1.1 and V3.3, and one strain (H50) showed activity against both C1 and V3.3. Interestingly, out of the 26 strains with antimicrobial activity, 19 strains produced bacteriocins as revealed by the complete inactivation of their supernatant fluids against the most sensitive indicator after trypsin treatment compared to the controls (Fig 1). Based on the strongest and stable antagonistic activity (inhibitory zone diameter ≥ 9 mm) against at least two indicator bacteria (Fig 1), six strains, which were H9, H18, H51, H61, H77 and H108, were selected for further study.

Effect of physiochemical factors on activity of crude bacteriocin extracts: The effect of physiochemical factors on the activity of crude bacteriocin extracts is presented in Table 1. In short, the crude bacteriocin extracts were completely inactivated by proteolytic enzymes, proteinase K and trypsin, indicating a proteinaceous nature of the inhibitory compounds, called bacteriocins. In contrast, the treatment with lipase or α-amylase was found to have little or no effects on the bacteriocin activity (Table 1), which suggested the absence of a lipid and carbohydrate moiety in the bacteriocins in this study.

The experiments assessing the effect of temperature on the activity of crude bacteriocin extracts showed that 70-100% of the antimicrobial activity was retained after treatment at 60 °C for 30 min (except for strain H77) (Table 1). Moreover, the crude bacteriocin extracts from the strain H108 retained nearly 30% activity after 30 min at 100 °C but was completely inactivated by autoclave conditions. The experiments testing the effect of pH on antimicrobial activity showed that it was relatively stable at pH 4-10 for all six strains (Table 1). The crude bacteriocin extracts from strains H18, H51 and H108 remained more than 40% activity even at pH 12.

Identification and phylogenetic analysis of six selected strains: Morphological and culture-based studies showed that all six strains are facultatively anaerobic Gram-negative bacteria with rod-shaped cells. Further

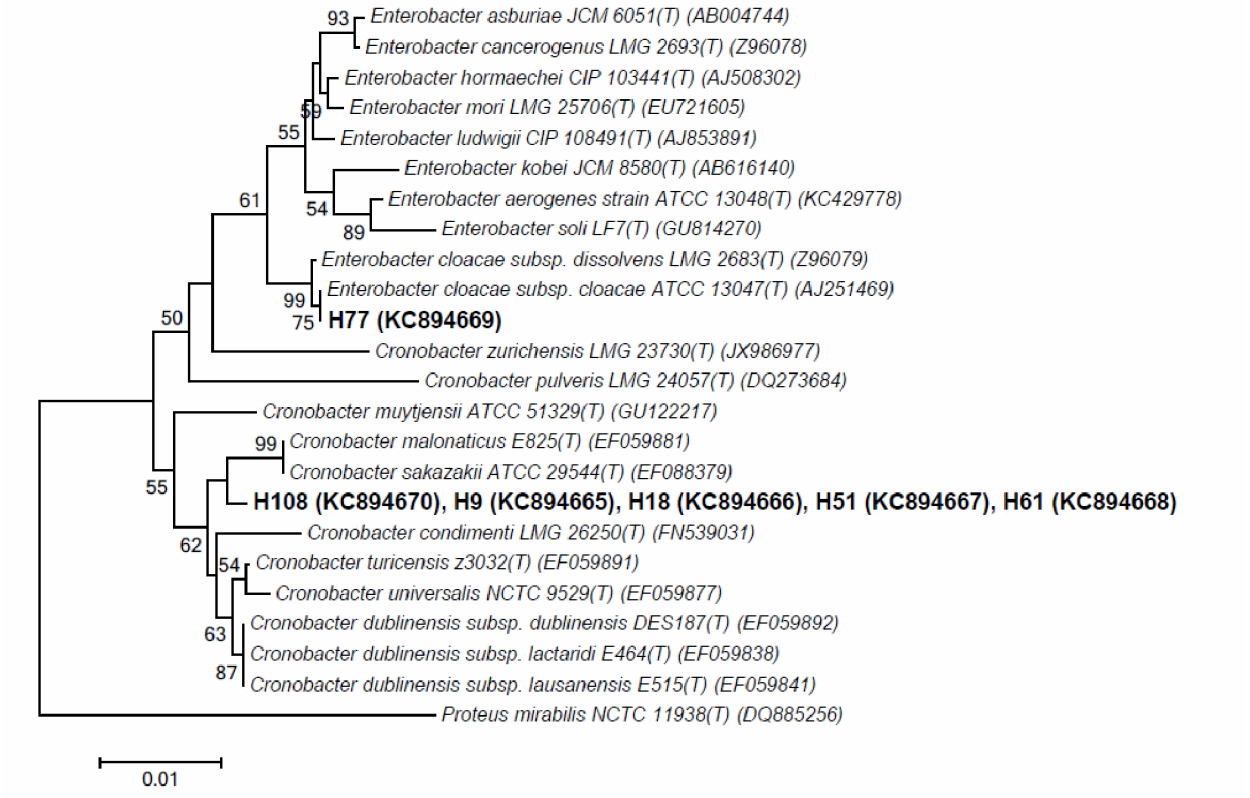

Figure 2 Neighbor joining phylogenetic tree based on comparative analysis of 16S rRNA gene sequences showing relationships between strains H77, H9, H18, H51, H61 and H108 and type strains of *Enterobacter* and *Cronobacter* species. GenBank accession numbers are shown in parentheses. Percentage of replicate trees at least 50% in bootstrap test (1000 replicates) is shown on the branches. The scale bar indicates number of substitutions per nucleotide position. (T) stands for type strains. *Proteus mirabilis* was used as out-group.

identification was carried out by 16S rRNA gene amplification and sequencing. Gene sequences were submitted to GenBank with the Gene Accession numbers KC894665, KC894666, KC894667, KC894668, KC894669 and KC894670 for the strains H9, H18, H51, H61, H77 and H108, respectively. The results from 16S rRNA gene sequencing of five strains H9, H18, H51, H61 and H108 revealed 100% homology to *Cronobacter sakazakii* strain Lc10g (Gene Accession number JQ963902), 99.6% to *Cronobacter malonaticus* PHLTA-12 (FN401344), 99.4% to the type strain ATCC 29544 of *Cronobacter sakazakii* (EF088379) and 99.3% to the type strain E825 of *Cronobacter malonaticus* (EF059881) while the strain H77 had 100% homology to *Enterobacter cloacae* AB2 (JX188069), 99.8% to the type strain ATCC 13047 of *Enterobacter cloacae* (AJ251469), 99.2% to *Enterobacter kobei* BM0593 (JQ680938) and 98.1% to *Enterobacter cowanii* 6L (DQ919062).

In addition, phylogenetic analysis of the 16S rRNA gene sequences of six isolates from otter clam showed significant differences with type strains of all species within the genera *Cronobacter* and *Enterobacter* (Fig 2). The strain H77 along with the type strain of *E. loacae* was well distinguished with all other *Enterobacter* species including *E. kobei*, suggesting the strain H77 could belong to *E. cloacae*. However, five strains, which were H9, H18, H51, H61 and H108, along with type strains of *C. sakazakii* and *C. malonaticus* showed the same clustering in the phylogenetic tree, which required further analysis for a species-level differentiation.

Therefore, the *rpoB* genes of the strains H77 and H108 were further amplified and sequenced with the Gene Accession numbers KF028398 and KF028399, respectively. The phylogenetic analysis of the *rpoB* gene sequences of these two strains with type strains of species within the genera *Cronobacter* and *Enterobacter* confirmed H77 belonging to *E. cloacae* and revealed H108 as *C. sakazakii*, which was well separated with bootstrap value of 99% from *C. malonaticus* in the tree (Fig 3).

Production of bacteriocins by C. sakazakii H108, and their effect on V. parahaemolyticus V1.1: The maximum bacteriocin production activity (inhibitory zone diameter of 20 mm or bacteriocin activity of 6400 AU/ml) of the strain *C. sakazakii* H108 was recorded after 33 h of growth when tested against *V. parahaemolyticus* V1.1 in TSB medium (Fig 4A). The bacteriocin production activity decreased significantly after 36 h of growth to a half of maximum level after 42 h.

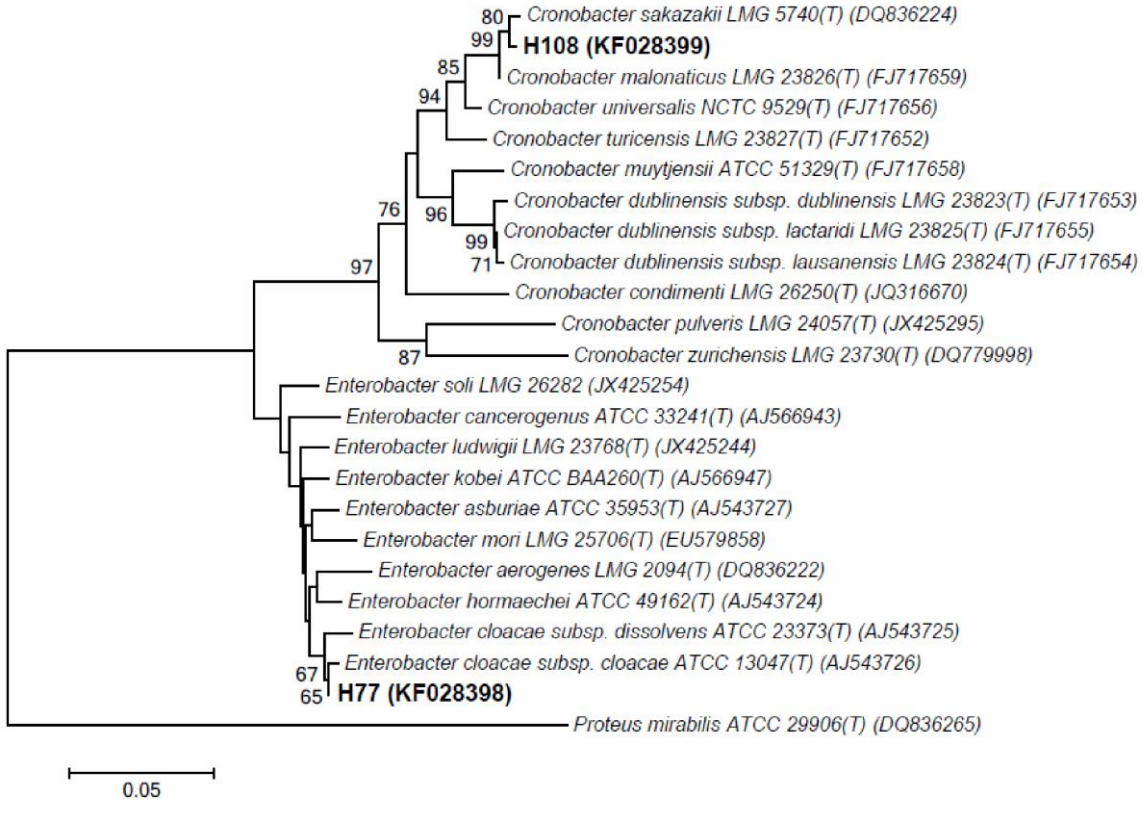

Figure 3 Neighbor joining phylogenetic tree based on comparative analysis of *rpoB* gene sequences showing relationships between strains H77 and H108 to closely related species. GenBank accession numbers are shown in parentheses. Percentage of replicate trees at least 50% in bootstrap test (1000 replicates) is shown on the branches. The scale bar indicates number of substitutions per nucleotide position. (T) stands for type strains. *Proteus mirabilis* was used as outgroup.

The addition of crude bacteriocin extracts (obtained from a 33-h-old culture of *C. sakazakii* H108) to 4-h-old cultures of *V. parahaemolyticus* V1.1 (OD_{600} ≈ 0.4) repressed cell growth over the next 10 h (Fig 4B). In particular, the bacteriocin extracts of H108 at the concentrations of 400, 800 and 1600 AU/ml reduced 15.15%, 47.27% and 72.12% growth of indicator bacteria after 10 h of culture compared to the untreated control, respectively.

Discussion

This study represents the first evidence of bacteriocin production by bacteria associated with otter clam (*L. philippinarum*). Among the total of 128 bacterial isolates, 19 (15%) were found to produce bacteriocins against at least one of three shrimp pathogenic *Vibrio* strains. Six strains (H9, H18, H51, H61, H77 and H108) with the highest bacteriocin production activity were selected for further defining the effect of physiochemical factors on the activity of crude bacteriocin extracts. The antimicrobial activity of crude bacteriocin extracts from these strains was completely inactivated after proteinase K and trypsin treatment but not with α-amylase treatment. Balcázar et al. (2010) obtained similar results in the case the cell-free culture supernatants of thirteen isolates from seahorses (*Hippocampus guttulatus*), which were also treated with proteinase K and trypsin. The results showed that the cell-free culture supernants from three strains (HG-14F, HG-12F and HG-3F) were inactivited by proteinase K, whereas only two strains were inactivated by trypsin. It is expected that proteinaceous substances are differentially cleaved by enzymes such as trypsin, proteinase K and α- chymotrypsin according to the protein structure (Riley and Wertz, 2002). In addition, the results are consistent with a previous study where two *Bacillus subtilis* strains, L10 and G1, retained 71-83% antagonistic activity against two shrimp pathogens, *V. harveyi* and *V. parahaemolyticus*, after α-amylase treatment compared to untreated samples (Zokaeifarl et al., 2012).

The sequencing and phylogenetic analysis of the 16S rRNA and *rpoB* genes resulted in the identification of one strain (H77) as *Enterobacter cloacae*

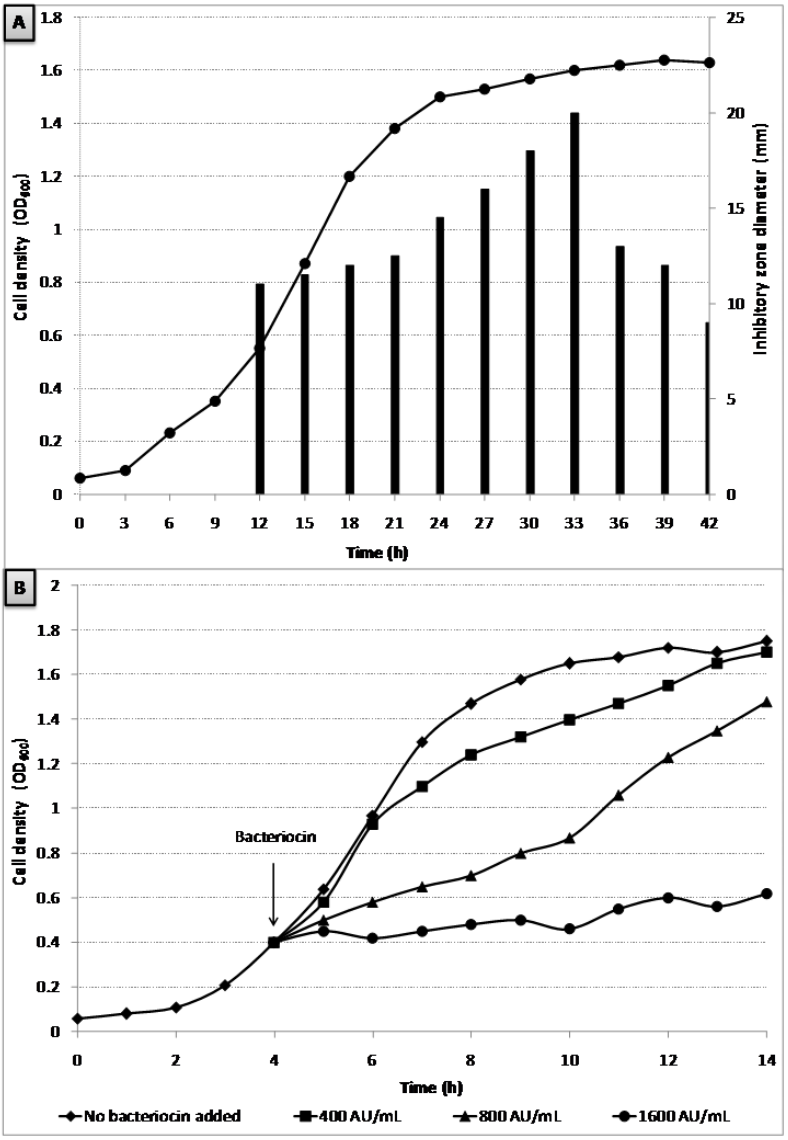

Figure 4 (A) Production of bacteriocins by *Cronobacter sakazakii* H108 (■) recorded as diameter of inhibition zone (mm), in TSB medium. Changes in optical density of H108 (●) are indicated. (B) Effect of crude bacteriocins produced by H108 on growth of V*ibrio parahaemolyticus* V1.1. The arrow indicates the point at which the bacteriocins were added.

Table 1 Effect of enzymes, temperature and pH on the activity of crude bacteriocin extracts against *V. parahaemolyticus* V1.1

	Inhibitory zone diameter in mm (residual activity, %)[a]					
Strain / **Treatment**	**H9**	**H18**	**H51**	**H61**	**H77**	**H108**
Control 1 (55°C, 3 h)	11(100)	14(100)	16(100)	13(100)	13(100)	16(100)
Proteinase K (55°C, 3 h)	0	0	0	0	0	0
Trypsin (55°C, 3 h)	0	0	0	0	0	0
Control 2 (37°C, 2 h)	10(100)	15(100)	17(100)	12(100)	14(100)	16(100)
Lipase (37°C, 2 h)	8.5 (85)	13(86.6)	13(76.5)	12(100)	13(92.9)	15(93.4)
Control 3 (20°C, 2 h)	10.5(100)	12.5(100)	16(100)	12 (100)	17(100)	10.5(100)
α- amylase (20°C, 2 h)	9.5(90.5)	13(104)	12(75)	9 (75)	12.5(73.5)	8(76.2)
Control 4 (30°C, 30 min)	12(100)	13(100)	13(100)	15(100)	10(100)	17(100)
60°C, 30 min	11(91.7)	10(76.9)	13(100)	11(73.3)	0	12(70.6)
100°C, 30 min	0	0	0	0	0	5(29.4)
121°C, 15 min	0	0	0	0	0	0
Control 5 (pH=7, 30 min)	10(100)	8(100)	10(100)	11(100)	13 (100)	14(100)
pH=2, 30 min	7(70)	0	0	4(36.4)	6(46.1)	4(28.6)
pH=4, 30 min	8(80)	7(87.5)	8(80)	6(54.5)	10(76.9)	7(50)
pH=6, 30 min	9(90)	10(125)	11(110)	8(72.7)	12(92.3)	16(114.3)
pH=8, 30 min	11(110)	8(100)	9(90)	8(72.7)	11(84.6)	13(92.9)
pH=10, 30 min	7(70)	6(75)	4 (40)	7 (63.6)	9 (69.2)	10 (71.4)
pH=12, 30 min	0	4(50)	4 (40)	0	0	6 (42.9)

[a] All presented zone diameters are an average of six results.

and the other strains as *Cronobacter sakazakii*. Previous taxonomic evaluation and current reclassification of the genera *Enterobacter* and *Cronobacter* based on multilocus sequence analysis (MLSA) indicated the presence of nine species recognized in each genus, in which *E. cowanii* has just been transferred into the novel genus *Kosakonia* as *K. cowanii* (Brady et al., 2013). In addition, the *rpoB* sequence analysis showed better resolution than 16S rRNA gene sequencing, which is in agreement with a previous study (Mollet et al., 1997). Therefore, *rpoB* may be used as a useful marker for bacterial identification in the Enterobacteriaceae family.

Species of the genera *Enterobacter* and *Cronobacter* are widely distributed in nature, but some of them can act as opportunistic human pathogens. For example, *E. cloacae* strains caused nosocomial bloodstream infections in the last decade (Mezzatesta et al., 2012) while *Cronobacter* species could lead to necrotizing enterocolitis, bacteraemia and meningitis (Yan et al., 2012). Because of little knowledge of their virulence-associated properties, further development to get a better understanding of the ecology and virulence of the *Cronobacter* and *Enterobacter* strains is required. If the six strains, which were H77, H9, H18, H51, H61 and H108, can be confirmed as non-pathogens towards humans and shrimp, they might be promising probiotics for potential applications against vibriosis in shrimp, as sensitive indicator bacteria in this study are potential shrimp pathogens of AHPNS. It has also been suggested that the gastrointestinal tract is a possible port of entry for *V. parahaemolyticus* and a target for protective methods using probiotic-containing feeds (Nguyen et al., 2013).

In contrast, if the six strains are shown to be pathogenic, otter clam *L. philippinarum* can be considered as a new source and vehicle of *E. cloacae* and *C. sakazakii* in the integrated food safety system from clam farming to human consumption. Only if the bacteriocins are not implicated in their pathogenicity, they might be purified and developed as an alternative antibiotic to control pathogens in aquaculture. Until now, no reports on bacteriocins produced by members of *Cronobacter* genus have been published whereas cloacin DF13 has been the only bacteriocin found in *E. cloacae*. This is a type of colicin, a bacteriocin made by *E. coli* which acts against other nearby *E. coli* to kill them with its 16S rRNase activity (De Graaf et al., 1986).

By now bacteriocins have been found in many major lineages of Eubacteria and some members of Archaea (Riley, 2009). Current progress in the research of bacteriocins and booming genomic data has led to develop the bacteriocin database: BACTIBASE (Hammami et al., 2007) and BAGEL (De Jong et al., 2010). The BAGEL tool helps to identify putative bacteriocins on the basis of conserved domains, physical properties and the presence of biosynthesis, transport and immunity genes in their genomic context. According to BAGEL bacteriocin database, more than 400 bacteriocins have been partially or fully characterized to date. They are produced by many genera in Actinobacteria, Firmicutes and Gammaproteobacteria. However, very few or none of members of other Proteobacteria, other bacteria phyla and Archaea have been shown to produce their bacteriocins.

Finally, we recorded the bacteriocin production by the strain *C. sakazakii* H108 during the growth and the effect of its crude bacteriocin extracts on the growth of the most sensitive indicator *V. parahaemolyticus* V1.1. Bacteriocin production activity increased by the time of growth to the highest values after 33 h of growth at the early stationary phase and then reduced at the end of fermentation likely due to degradation of the bacteriocin by extracellular proteolytic enzymes. According to Cascales and coworkers (2007), this trend is characteristic of primary metabolite production for bacteriocins produced by enteric bacteria. When the crude bacteriocin extracts of *C. sakazakii* H108 were added to the growing cultures of *V. parahaemolyticus* V1.1, cell growth of the indicator was inhibited significantly. Moreover, no viable cells of *V. parahaemolyticus* V1.1 were observed after 14 h in the

presence of bacteriocin extracts H108 using light microscopy (*data not shown*), suggesting that the inhibitory type of bacteriocins H108 is bactericidal.

As well as the search for new antimicrobials, a new resource or strain for the known bacteriocins is also important. This paper presents the first proof of bacteriocins produced by a member of the genus *Cronobacter* and also by bacteria associated with clam. The strains H9, H18, H51, H61 and H108 belonging to *Cronobacter sakazakii* and the strain H77 of *Enterobacter cloacae* expressed their highest bacteriocin production activity against *Vibrio parahaemolyticus* V1.1, which were isolated from AHPNS-infected tiger shrimps. The maximum bacteriocin production activity of 6400 AU/ml of the strain *C. sakazakii* H108 was recorded after 33 h of growth at the early stationary phase. The crude bacteriocins H108 at the concentration of 1600 AU/ml expressed their strong bactericidal activity against *V. parahaemolyticus* V1.1. Therefore, the research creates a paradigm for future studies of marine bacteriocins as novel drugs for animal health in general and for the biocontrol of vibrios in shrimp in particular.

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บทคัดย่อ

การตรวจคัดกรองหาสารที่มีฤทธิ์ต้านจุลชีพที่คล้ายกับแบคทีริโอซินในการต้านเชื้อ Vibrio ที่ก่อโรคในกุ้ง และการจำแนกชนิดของเชื้อแบคทีเรียจากน้ำเค็มที่แยกได้จากหอย *Lutraria philippinarum*

*Thu Thuy Pham Thi Hong Nhi Ho Van Duy Nguyen**

การศึกษานี้มีวัตถุประสงค์เพื่อแยกเชื้อแบคทีเรียน้ำเค็มจากหอย *Lutraria philippinarum* เพื่อคัดกรองหาสารมีฤทธิ์ต้านจุลชีพที่คล้ายกับแบคทีริโอซินในการต้านเชื้อ *Vibrio* ที่ก่อโรคในกุ้ง และจำแนกแบคทีเรียเหล่านี้ด้วยวิธีทางอณูชีววิทยา จากแบคทีเรียที่แยกได้ทั้งหมดจำนวน 128 ชนิด มีจำนวน 19 ชนิด (15%) ที่สามารถผลิตแบคทีริโอซินต้านอย่างน้อยหนึ่งในสามสายพันธุ์ของเชื้อ *Vibrio* ที่ก่อโรคได้ มีเชื้อ 6 สายพันธุ์ที่สามารถผลิตแบคทีริโอซินได้ในระดับสูงสุด ได้แก่ H9, H18, H51, H61, H77 และ H108 ฤทธิ์ต้านจุลชีพจากสารสกัดแบคทีริโอซินแบบหยาบจากเชื้อเหล่านี้ถูกยับยั้งโดยสมบูรณ์หลังจากใส่ proteinase K และ trypsin ลงไป นอกจากนี้ยังมีคุณสมบัติไวต่อความร้อนแต่ค่อนข้างเสถียรที่ค่าความเป็นกรด-ด่าง 4-10 การวิเคราะห์หาลำดับพันธุกรรมและสายสัมพันธ์ทางวิวัฒนาการของยีน 16S rRNA และ *rpo*B ทำให้สามารถจำแนกสายพันธุ์หนึ่ง (H77) ได้เป็น *Enterobacter cloacae* และอีกสายพันธุ์หนึ่งเป็น *Cronobacter sakazakii* งานวิจัยนี้ได้แสดงหลักฐานชิ้นแรกว่าแบคทีริโอซินสามารถถูกผลิตจากสมาชิกในสกุล *Cronobacter* และโดยแบคทีเรียที่มีความเกี่ยวข้องกับหอย งานวิจัยได้นำเสนอข้อมูลที่ให้การสนับสนุนการศึกษาความหลากหลายของแบคทีริโอซินอื่นๆ เพิ่มเติมในแบคทีเรียจากน้ำเค็ม และศักยภาพที่จะเป็นโปรไบโอติคหรือสารทดแทนยาปฏิชีวนะเพื่อใช้ในการเลี้ยงสัตว์น้ำอย่างยั่งยืน

คำสำคัญ: สารต้านจุลชีพ แบคทีริโอซิน *Cronobacter* โปรไบโอติก กุ้ง *Vibrio*

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